1994

The Effects of Contaminated Sediment on the Epidermal Goblet Cells of the Mummichog, Fundulus heteroclitus

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The Effects of Contaminated Sediment on the Epidermal Goblet Cells of the Mummichog, *Fundulus heteroclitus*

A Thesis
Presented to
The Faculty of the School of Marine Science
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In Partial Fulfillment
Of the Requirements for the Degree of
Master of Arts

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by
Laurent C. Mézin
1994

This thesis is submitted in partial fulfillment of
the requirements for the degree of

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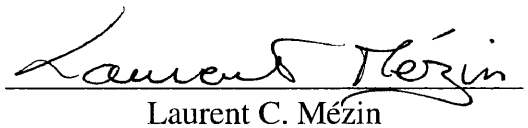

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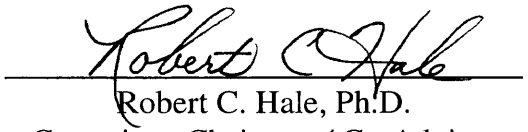

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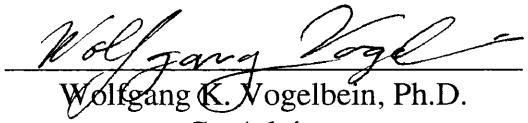

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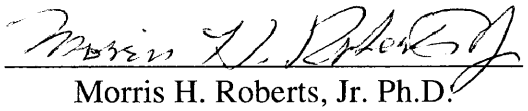

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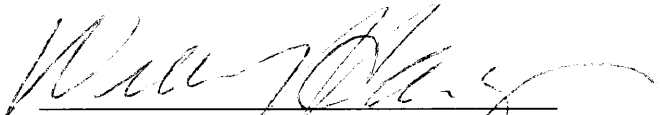

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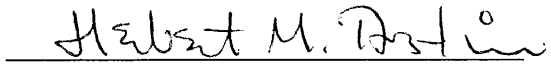

Herbert M. Austin, Ph.D.

Dedication

To my parents, Michel and Patricia Mézin.
In partial fulfillment of their hopes, expectations
and years of tender loving care.

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Abstract

Secretion of mucus by epidermal goblet cells is an important first line of defense for teleosts. It protects them against many of the biological, physical and chemical insults they encounter in their environment. This project monitored changes in hemoglobin concentration in epidermal mucus and in the density, diameter and mucus quality of epidermal goblet cells in the mummichog, *Fundulus heteroclitus*, following exposure to creosote-contaminated sediment. Male fish (200) were exposed for 13 days in flow-though aquaria to either an uncontaminated reference sediment or contaminated sediment containing 30% Elizabeth River sediment. Fish were sampled on Days 0, 3, 7 and 13. The hemoglobin content of their mucus was assessed using a commercial hemoglobin test strip. Their condition index was determined and a portion of their ventral skin was mounted after sequential staining by alcian blue and periodic acid Schiff's reagent. The proportion of the mucin types present in the goblet cells, differentiated by the stains, was determined using light microscopy (600x). The aromatic compound concentrations in test aquaria effluents decreased significantly during the experiment, probably as a result of a reduction in resuspension of the sediment by the fish. The condition index was lower and the mortality rate and occurrence of epidermal lesions were higher in the treated fish than in the control fish. The hemoglobin content in the epidermal mucus of treated fish was significantly higher than in control fish. Significant reductions in both size and density of goblet cells observed in treated fish suggested a mucus secretion rate exceeding its production rate. Significant variations in mucin types occurred in both treatments, most likely as a result of the transfer of the fish from the holding tank to the experimental aquaria. Significant changes in mucin types between treatments did not occur until Day 13 and are not believed to be directly related to the creosote present in the treatment sediment.

The Effects of Contaminated Sediment on the Epidermal Goblet Cells of the Mummichog, *Fundulus heteroclitus*

Introduction

In fish, the first barriers against environmental stressors are the skin, gills and alimentary tract. The skin, one of the largest organs in fishes, has a variety of functions, ranging from camouflage (Tave *et al.* 1991) to osmoregulation (Shephard 1981). The defense against physical, biological and chemical insults is perhaps the skin's most important function. The skin is challenged by, and reacts to organisms such as viruses and parasites, as well as changes in water chemistry and anthropogenic pollutants. Should the skin fail in its defensive role, the health of the fish may be compromised.

Some stressors can lead to injury of the skin, ranging from mild superficial abrasions resulting in simple inflammation to deep ulcerative lesions that expose the underlying muscle or even the peritoneal cavity and that can lead to death. Inflammation is "the continuum of vascular exudative changes in tissue elicited by the effect of irritants on blood vessels" (Cheville 1983). It results in increased blood flow and leakage of blood components into the area. Hemorrhage often accompanies tissue injury and inflammation. The red blood cells often lyse, releasing free hemoglobin which can migrate from the inflamed tissue to the mucus (Smith and Ramos 1976).

Mucus, which covers the outer surface of the skin, is produced by the epidermal goblet cells and provides a variety of host defense mechanisms. Continuous synthesis and secretion of mucus by goblet cells provide a mechanical means of

protection against parasites (Pickering 1974). The mucus of some fishes has been reported to contain ichthyotoxins (Nair *et al.* 1982). Non-specific substances with defense activities such as antibacterial compounds including lysozyme, hemogglutinins, proteases and trypsin have been found in the mucus of some teleosts (Hjelmeland *et al.* 1983; Austin and McIntosh 1988; Fouz *et al.* 1990; Braun *et al.* 1990). Specific antibodies have also been found in fish mucus (Ingram 1980). Mucus binds to metals such as copper, cadmium, mercury, aluminum and zinc (Miller and Mackay 1982; Pärt and Lock 1983; Handy and Eddy 1989; Handy and Eddy 1990) and has been shown to be an important excretion pathway for metals as well as for hydrocarbons and their metabolites (Varanasi and Markey 1978; Varanasi *et al.* 1978; Pärt and Lock 1983; Van Veld *et al.* 1984; Handy and Eddy 1989; Handy and Eddy 1990).

During exposure of fish to environmental stressors, the characteristics of mucus may change in a variety of ways depending on the nature and severity of the stressor. Numerous field and laboratory studies describe the effects of metals (Mueller *et al.* 1991; Battaglini *et al.* 1993; Prasad and Shil 1993), pH (Mc Cahon *et al.* 1987; McDonald *et al.* 1991a), ectoparasites (Pottinger *et al.* 1984), bacterial gill disease (Speare *et al.* 1991; Ferguson *et al.* 1992), hydrocarbons (Prasad 1987; Davison *et al.* 1993) and other agents (Bolognani Fantin *et al.* 1984; Mallatt 1985; Burton and Everard 1991; Speare and Mirsalimi 1992) on the quantity and quality of mucus and on the diameter and density of mucus-secreting goblet cells. For example, Battaglini *et al.* (1993) demonstrated an increase in both mucus secretion and sulphated mucosubstances in fish exposed to cadmium. Ferguson *et al.* (1992) showed an

increase in neutral mucosubstances in fish challenged with bacterial gill disease (BGD) or ammonia, but a decrease in sulphated mucosubstances, in trout exposed to BGD. Solanki and Benjamin (1982) subjected fish to increased salinity and demonstrated a decrease in both sulphated mucosubstances and density of goblet cells, a possible indicator of increased mucus production.

Direct quantification of total mucus production is possible by monitoring the sialic acid content in either the tissue or the water (Eddy and Fraser 1982; McDonald *et al.* 1991b). These methods require both a static system, for the accumulation of mucus and a species whose mucus contains sialic acid (Nakagawa *et al.* 1988). Changes in goblet cell size and density is also a widely accepted means of quantitating mucus production (Pickering 1974).

Differences in the density of epidermal goblet cells of fishes occur between sexes and even locations on the body (Pickering 1974), although the synthesis of mucus apparently does not change between species (Henrikson and Matoltsy 1968). Goblet cells begin to differentiate in the *stratum germinativum*, a basal layer of columnar cells in the epidermis and migrate to the surface, through the squamous epithelium (Yasutake and Wales 1983; Roberts 1989). They produce mucus and progressively increase in size. The mucus is stored in membrane-bound droplets which often coalesce, especially near the apex of the cell, before fusing with the plasma membrane of the goblet cell. Fusion of membrane-bound droplets with the plasma membrane allows the mucus to be released on the surface of the skin

(Henrikson and Matoltsy 1968). Following mucus secretion, the epidermal goblet cell of fishes, a holocrine gland, dies and is sloughed (Hibiya 1982; Yasutake and Wales 1983; Iger *et al.* 1988).

The basic mucus composition in teleosts seem to be similar to that of other animal species (Handy 1989). Its main components are mucins. Mucins consist of a filamentous protein core with short polysaccharide side chains. The protein core is synthesized by the polyribosomes of the rough endoplasmic reticulum (RER) and the linkage region sugars are added within the RER before transport to the Golgi complex. Within the Golgi complex, the polysaccharide side chains are elongated and further modified (Horwitz and Dorfman 1968; Mittal *et al.* 1980; Ghadially 1988). In mucus ready for secretion, the side chains, two to twenty monosaccharides long, may be branched or linear, sulfated or sialated, both or neither. These characteristics provide mucus with variable histochemical properties (Verdugo 1990). This side chain elongation can be discerned histochemically as a difference in staining properties (from neutral to acidic) of goblet cells as they migrate to the superficial layers of the epidermis (Bolognani Fantin *et al.* 1984). The sulfated and sialated residues also give the mucins a strong polyanionic charge at neutral pH. Within the cell, this charge is shielded by cations such as Ca^{2+}.

Verdugo (1990) suggested that exocytosis of mucus is driven by electrostatic interactions, as the cations diffuse into the water when the vesicle containing mucus fuses with the plasma membrane and opens to the surface. As a consequence, both the ionic charge of the mucus and the ions present play an important role in the

viscosity of the mucus on the epidermal surface. Solanki and Benjamin (1982) noted that an increase in acidity of mammalian mucus caused an increase in its viscosity.

Histochemical methods to differentiate between the mucin types have been known for over 20 years (Jones and Reid 1973). Sequential staining with alcian blue 8GX and periodic acid Schiff's reagent (AB/PAS) can be used to distinguish goblet cell sub-populations based on the staining affinities of the mucins. When the pH of the alcian blue is 2.5 (AB2/PAS), acidic mucins stain blue and neutral mucins stain red. At a pH of 1.0 (AB1/PAS), sulfated mucins stain blue and non-sulfated mucins stain red (Table 1). Goblet cells containing both types of mucins at a given pH will have a mixed reaction and their contents will stain purple (Solanki and Benjamin 1982; Battaglini *et al.* 1993).

Eisler (1986) stated that teleost species used for toxicity testing "...should cover a wide geographic range, be abundant throughout most of that range, be easy to collect, be adaptable to laboratory conditions, be representative of other teleosts in sensitivity to chemicals, and be of economic or recreational importance." The mummichog, *Fundulus heteroclitus* (L.), meets all of these requirements. It is found in schools of varying sizes throughout the mid-Atlantic region, close to shore and in the bays, estuaries and tidal creeks. The mummichog has a very limited summer

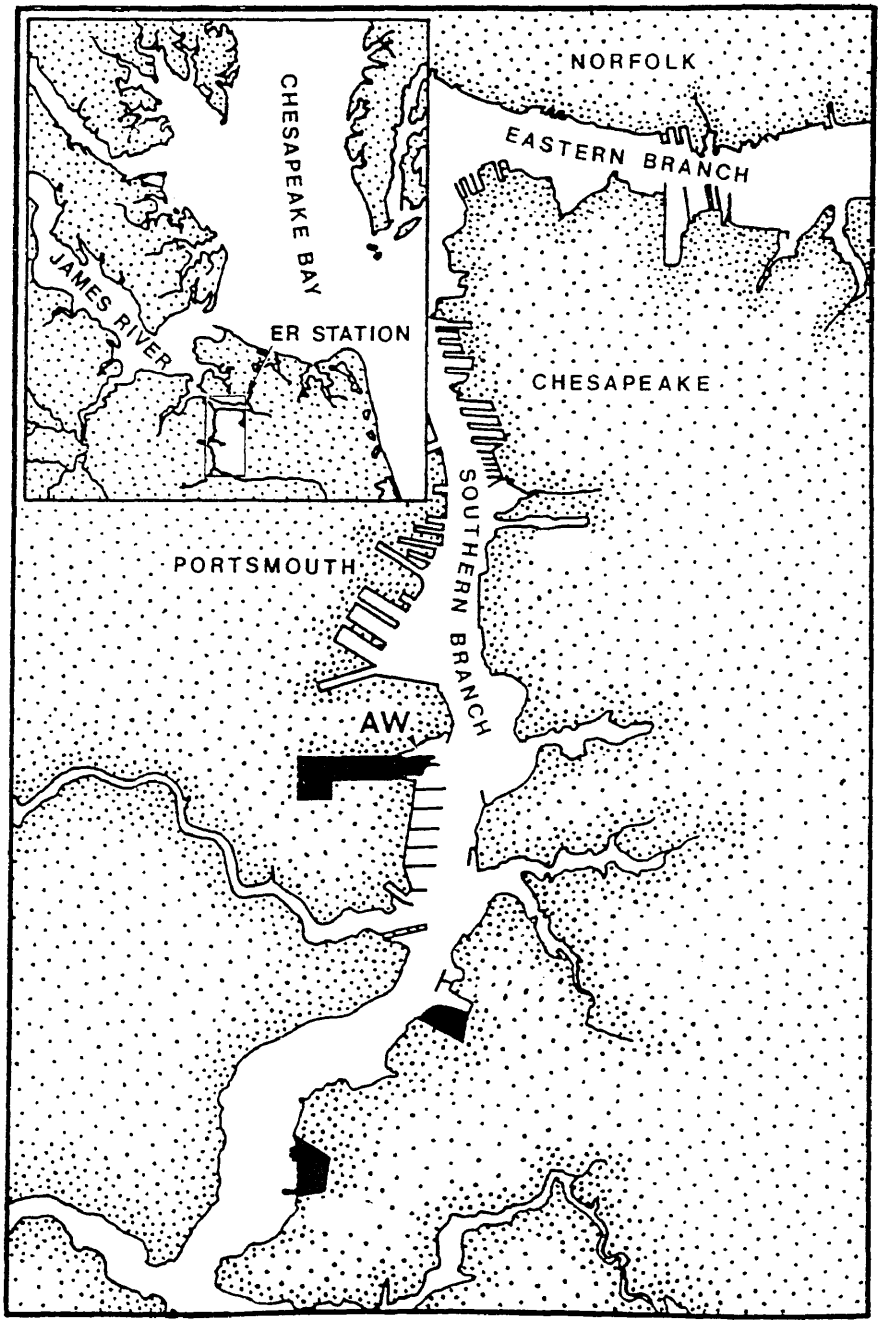
Table 1. Histochemical methods for mucin differentiation (Solanki *et al.* 1982; Battaglini *et al.* 1993).

Method:	Mucin Type:	Stain:
$AB_{1.0}$/PAS	Sulfated:	Blue.
	Non-Sulfated:	Red.
	Mixed-sulfated:	Purple.
$AB_{2.5}$/PAS	Acid	Blue.
	Neutral:	Red.
	Mixed-acid:	Purple.

home-range (Lotrich 1975; Hoff 1985) and a high tolerance to temperature and salinity fluctuations. Tidal cycles in Maine salt marshes expose mummichog to rapid temperature and salinity changes (Abraham 1985). For example, summer temperatures can fluctuate from 15 to 30 °C (Abraham 1985). It has one of the highest total biomass productions for natural populations in fishes (Meredith and Lotrich 1979; Abraham 1985). In the southern Chesapeake Bay, the mummichog matures at one year, at which time its total length typically exceeds 32 mm for males and 38 mm for females (Abraham 1985). Sex specific coloration also allows for easy identification of the sexes. The mummichog is easily captured using baited minnow traps. It is a valuable resource species of the small bait industry and its abundance and wide-spread distribution make it an important part of the estuarine food web. The mummichog has been used extensively in research and is easily maintained in the laboratory (Eisler 1986). It has been known to burrow down into the mud, to a depth of 2 cm (Abraham 1985). This behavior would put it in intimate contact with contaminants found in the sediment. Vogelbein *et al.* (1990) reported high prevalences of hepatic neoplasms in mummichog from a site in the southern branch of the Elizabeth River, Virginia, which is heavily contaminated with polycyclic aromatic hydrocarbons (PAHs) derived from creosote.

The Elizabeth River (ER) is one of Virginia's most polluted estuary (Hargis *et al.* 1984; Huggett *et al.* 1984). It is a tributary of the James River, which opens into the lower Chesapeake Bay (Fig. 1). Lined with a variety of industries and military

Figure 1. Map of the Chesapeake Bay. The treatment sediment was taken from the river, directly adjacent to the Atlantic Wood Inc. wood treatment facility (AW).


NORFOLK
EASTERN BRANCH
CHESAPEAKE BAY
JAMES RIVER
ER STATION
CHESAPEAKE
SOUTHERN BRANCH
PORTSMOUTH
AW

facilities, as well as the cities of Norfolk and Portsmouth, it has long been the site of industrial and urban pollution. The sediments of the river contain a variety of contaminants, such as aliphatic and aromatic hydrocarbons, polychlorinated biphenyls heavy metals and tributyltin (Buchman *et al.* 1992, Espourteille *et al.* 1993).

PAH contamination of the southern branch of the river is especially severe due to spills from several wood treatment facilities operating along its shores throughout this century (Huggett *et al.* 1992). These facilities have traditionally used creosote to pressure-treat timbers. Creosote is produced by high temperature carbonization of bituminous coal. This typically results in a functionally defined mixture composed mostly of PAHs and heterocyclic analogues (Nestler 1974). These aromatic compounds confer upon creosote its biocidal properties.

Analyses of sediments from the Elizabeth River reveal that 14 PAHs are present in high concentrations (up to g / kg sediment dry wt) at over 90 % of the 30 stations surveyed throughout the river (Table 2) (Huggett *et al.* 1992). Hundreds more have been detected. The Atlantic Wood Station, in particular, is heavily contaminated by aromatic compounds from a nearby wood treatment plant.

In contrast, the York River (YR) has low concentrations of these contaminants and has been chosen as a reference estuary by several researchers (Hargis *et al.* 1984; Faisal *et al.* 1991). Kings Creek, a tributary of the Severn River, also has low contaminant levels (ca. 3 mg PAH / kg sediment dry wt) (Van Veld, P., personal communication).

Table 2. 14 PAHs present at high concentrations in sediments collected from 30 sampling stations in the Elizabeth River, Virginia (Huggett *et al.* 1992).

Compound	Maximum observed Concentration (μg/kg sediment)	Mean Concentration (μg/kg sediment)
Phenanthrene	2,400,000	40,000
Fluoranthene	1,000,000	24,000
Pyrene	620,000	17,000
Anthracene	1,300,000	10,000
Chrysene	180,000	6,000
Benzofluoranthene	110,000	5,700
Benzo(b)fluorene	120,000	4,600
Benzo(a)fluorene	120,000	4,500
Benzo(a)anthracene	140,000	4,300
Benzo(a)pyrene	50,000	2,800
Benzo(e)pyrene	56,000	2,500
Indeno(1,2,3-cd)pyrene	18,000	1,000
Benzo(ghi)perylene	14,000	870
Perylene	14,000	710
Total	6,170,000	124,000

Previous studies have shown that fish from the Elizabeth River exhibit higher prevalences of physiological and structural abnormalities such as lens cataracts (Hargis and Zwerner 1989), epidermal lesions (Hargis *et al.* 1984; Bender *et al.* 1988; Huggett *et al.* 1992), altered macrophage activity (Weeks *et al.* 1987; Weeks *et al.* 1990), neoplasms (Hargis *et al.* 1989; Vogelbein *et al.* 1990), altered hepatic ethoxyresorufin O-deethylase (Sved *et al.* 1992) and elevated glutathione S-transferase (Van Veld *et al.* 1991) than the same species in less polluted habitats. Some of these aberrations have been reproduced in the laboratory after exposure to ER sediments (Hargis *et al.* 1984; Weeks *et al.* 1990). Changes in cytochrome P-450 levels (Elskus and Stegeman 1989; Van Veld *et al.* 1992), altered macrophage chemiluminescent response (Kelly-Reay and Weeks-Perkins 1994) and necrosis of neurosensory cells such as taste buds (DiMichele and Taylor 1978) are some of the observed effects of PAH exposure in mummichog.

Objectives

The objectives of this study were to evaluate the effects of laboratory exposure to a creosote-contaminated ER sediment on epidermal goblet cell characteristics of the mummichog, *Fundulus heteroclitus*, by:

1. assaying the hemoglobin (Hb) levels in their epidermal mucus during a 13-day exposure;
2. a quantitative evaluation of goblet cell diameter, density and mucin type during a 13-day exposure.

Null Hypotheses

1. The Hb assay response will not differ, either between treatments or temporally, within treatments.
2. Goblet cell density and diameter will not differ, either between treatments or temporally, within treatments.
3. The proportion of the different mucin types will not differ, either between treatments or temporally, within treatments.

Materials and Methods

Mummichog:

Adult male mummichog (200 individuals, 70-90 mm total length) were collected from Kings Creek, Gloucester Point, VA. with baited minnow traps. The fish were treated for monogenetic trematode infections (*Gyrodactilus* sp.) by a 24 hour dip in a 2 mg/l concentration of Praziquantel (Sigma, St Louis, MO) (Zwerner, D., personal communication) and held in a 600 liter flow-through YR water tank for five weeks. The holding tank was vigorously aerated. A second Praziquantel treatment (10 mg/l, 3 hours) was administered on the fourth week. The fish were fed commercial food (Tetramarin®) twice daily. The food quantity (3 % body weight per day) was adjusted to ensure limited growth. Handling of the fish was minimized during the acclimation period. Growth was not monitored.

Sediment:

Sediments of similar particle size distributions were collected from Carter Creek, a tributary of the York River and from the Atlantic Wood station in the Elizabeth River. They were homogenized and stored in separate, closed plastic buckets in a cold room at ca. 4 °C. The water content was determined by drying and the total organic carbon content was determined by combustion of the dry sediment and the detection of the resulting CO_2 by a thermal conductivity detector. The analyses were performed by the nutrient laboratory of the Virginia Institute of Marine Science, Gloucester Point, Virginia. Treatment sediment was prepared by mixing control Carter Creek sediment with Elizabeth River sediment in a 70/30 mixture by volume.

Hemoglobin assay strips:

A commercial hemoglobin test strip, *Hema-combistix*® (Smith and Ramos 1976; AMES 1991) was applied directly to the mucus. These strips develop in 60 seconds and have a sensitivity of 0.15-0.62 mg Hb / liter, (i.e. the equivalent of 5-20 intact erythrocytes / μl), graded from "none" to "high". The test uses the peroxidase-like activity of hemoglobin and myoglobin to catalyze the reaction of diisopropyl-benzene dihydroperoxide and 3,3',5,5'-tetramethylbenzidine, resulting in a color shift from orange to green.

Histological solutions:

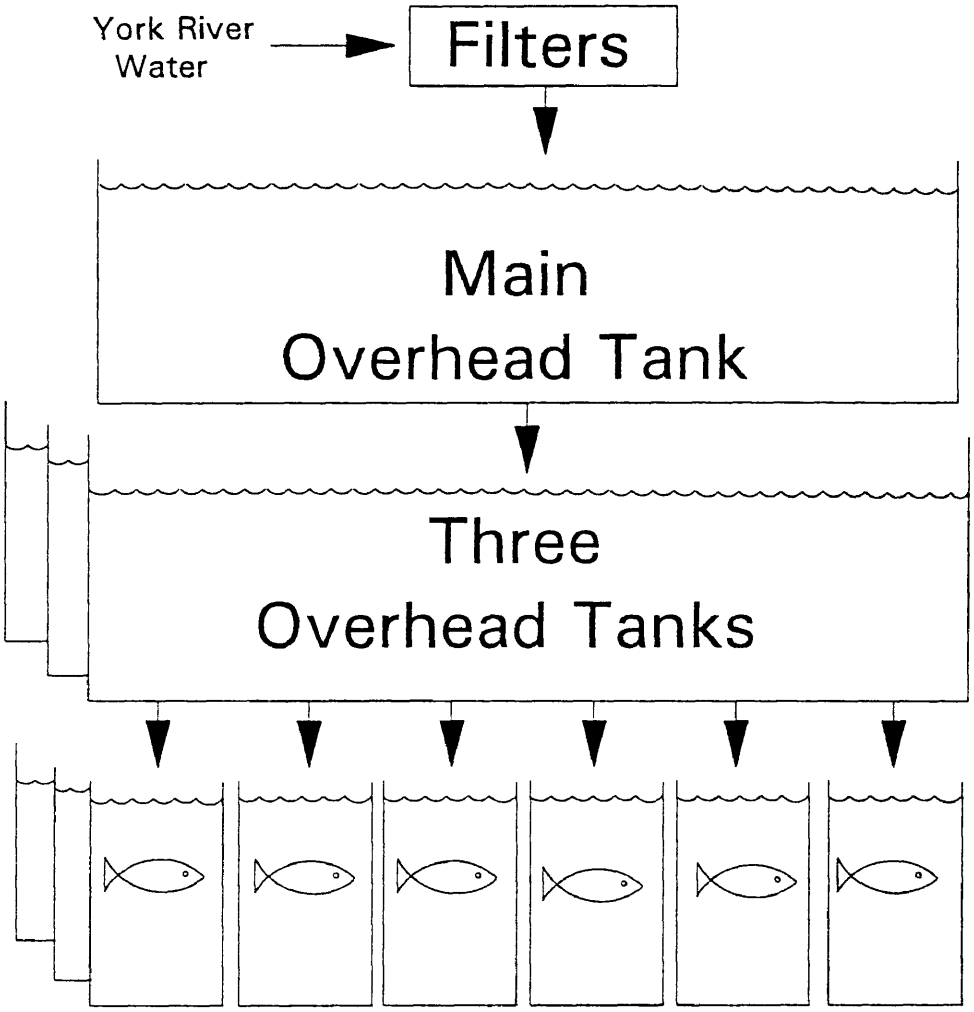
A 5% saline formaldehyde solution was used to fix the skin samples. The PAS-Alcian blue solutions were prepared according to Luna (1968). Methyl salicylate was used to clear the tissue samples. The mounting media was Permount®.

Exposure system:

Ten fish were placed in each of eighteen 40-liter glass aquaria. There were nine control and nine treatment aquaria divided into three sets for time series analyses. All aquaria were randomly placed behind individual opaque screens to minimize visual cues. Two liters of either control or treatment sediment were spread over an acrylic plastic "egg crate" grating (cell size ca. 1.3 x 1.3 cm, height 1.6 cm) placed at the bottom of each aquarium. The grating, level with the sediment surface, reduced resuspension of sediment into the water column by the fish. After the sediment settled, individual aquarium aeration and water flow were started and allowed to continue overnight to ensure adequate levels of dissolved oxygen.

Incoming York River water was filtered to 10 μm and held in a vigorously aerated main overhead tank. From this tank, the water was distributed to three secondary overhead tanks, each supplying six experimental aquaria (Fig.2). A flow rate of 500 ml/min. was established for each aquarium.

Figure 2. Schematic of the experimental design for exposure of mummichog, *Fundulus heteroclitus*, to creosote-contaminated sediment.


York River
Water
Filters
Main
Overhead Tank
Three
Overhead Tanks

Water quality:

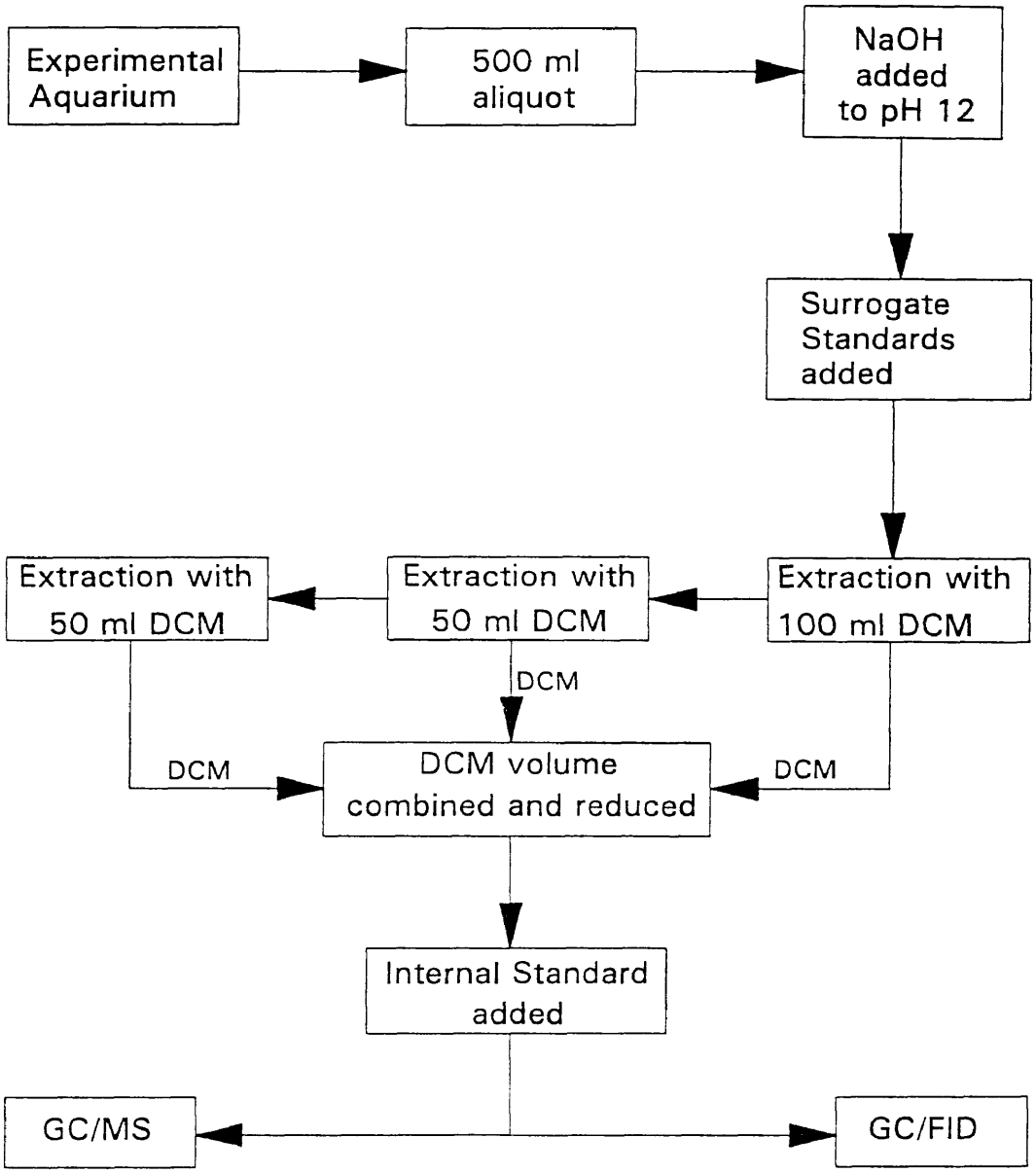
Temperature and salinity in each aquarium were measured daily. Dissolved oxygen (DO) was measured daily in each aquarium with a DO meter (YSI Model 50B). The oxygen saturation was calculated from the empirical formula (Hyer, unpublished data):

$$O_{2(sat'd)} = 14.6244 - (0.367134)T + (0.0044972)T^2 - (0.0966)S + (0.00205)TS + (0.0002739)S^2$$

with: T: temperature (°C) S: salinity (ppt)

The pH was measured twice a week in each aquarium with a pH-meter (Beckman Φ31).

Water samples for aromatic compound concentrations in the water columns and dissolved organic carbon determinations were collected 24 hours prior to each fish sampling period. The flow-thru siphon was allowed to clear of debris and 500 ml of aquarium effluent were sampled from it. The pH was raised to a value of 12 or greater by addition of NaOH. Known amounts of surrogate standards (1,1'-binaphthyl; D_8-naphthalene; D_{10}-acenaphthene and D_{12}-benzo(a)pyrene) were added to the water sample to quantify analyte losses occurring during the extraction. The aromatic compounds were sequentially extracted (Fig. 3) three times with a total of 200 mls (100+50+50 ml) of dichloromethane (DCM). Sodium chloride was added to reduce the formation of emulsions during extraction. The DCM volume was


Experimental Aquarium
500 ml aliquot
NaOH added to pH 12
Surrogate Standards added
Extraction with 50 ml DCM
Extraction with 50 ml DCM
Extraction with 100 ml DCM
DCM
DCM
DCM volume combined and reduced
DCM
Internal Standard added
GC/MS
GC/FID

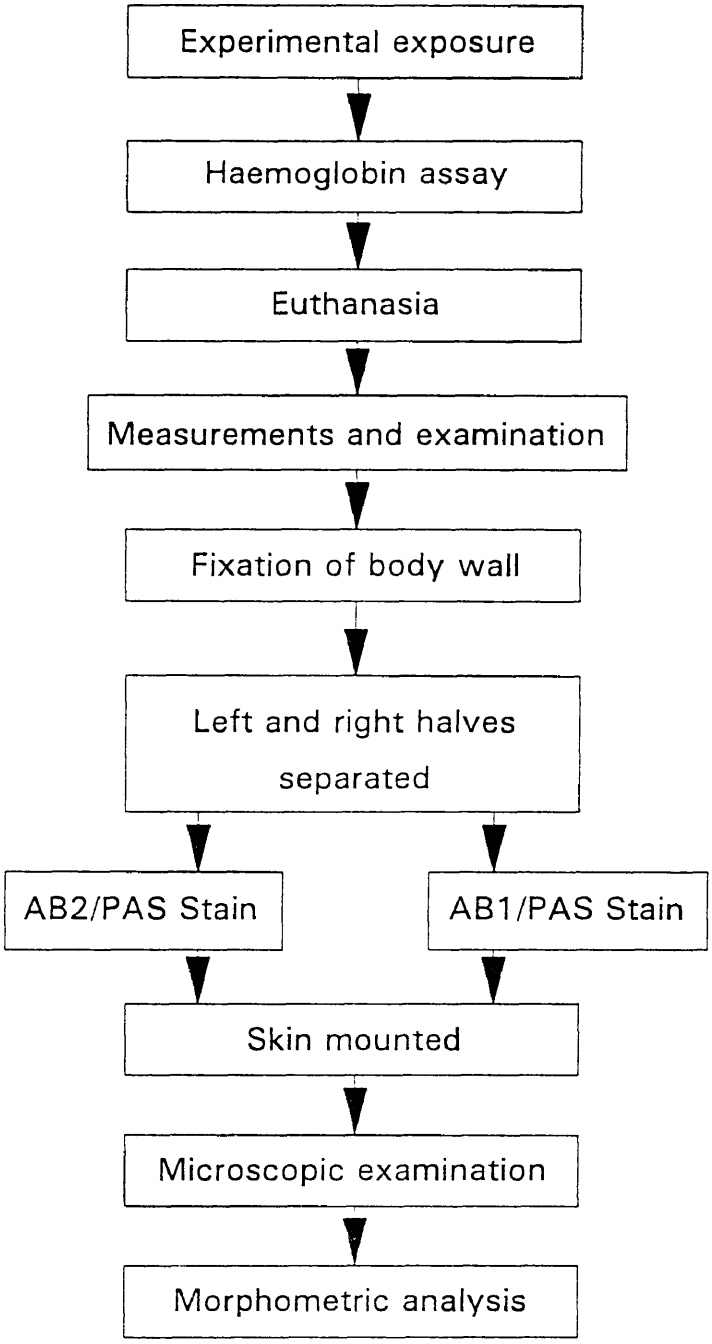
reduced by evaporation under nitrogen to ca. 0.1 ml and a terphenyl internal standard added immediately prior to gas chromatographic analysis. The analyses were performed on a Varian 3400 gas chromatograph equipped with a flame ionization detector. The initial column, injector and detector temperatures were 75, 260 and 320 °C, respectively. The column temperature was increased by 5 °C / min. to a final temperature of 310 °C. Samples containing only the standards dissolved in DCM were interspersed between the samples to determine the precise retention indices of the standards. The aromatic retention indices (ARI) of unknown peaks in the water samples were determined by interpolation using the ARI marker peaks and identified using an ARI library. A representative sample was further analyzed after a separate GC separation by a mass spectrometer (Finnigan Incos XL) in the electron ionization mode to confirm compound identification. Quantification of the identified aromatic compounds was achieved by comparing their peak areas to that of the internal standard and corrected for recovery.

Dissolved organic carbon samples were analyzed using a total organic carbon analyzer Model TOC-500 with a non-dispersive infrared detector. The analyses were performed by the nutrient laboratory of the Virginia Institute of Marine Science, Gloucester Point, Virginia.

Fish sampling:

On Day 0, twenty fish were processed (Fig. 4). For statistical purposes, they were randomly separated into two equal groups. Each group was treated as an

Figure 4. Schematic of the methods used to process mummichog following exposure to creosote-contaminated sediment.


Experimental exposure
Haemoglobin assay
Euthanasia
Measurements and examination
Fixation of body wall
Left and right halves separated
AB2/PAS Stain
AB1/PAS Stain
Skin mounted
Microscopic examination
Morphometric analysis

experimental aquarium and designated as C-0 (Control aquarium at Day 0). On subsequent sampling days, three control and three treatment aquaria were chosen using a random number generator and the fish therein processed. The fish were removed from the aquaria with a net and hemoglobin test strips immediately applied to the mucus on their skin, one on each side of the caudal peduncle. The strips were read after 60 seconds. The responses were given a value from 0 to 4 (0: none; 1: trace; 2: low; 3: moderate; 4: high) and the higher of the two responses for each fish recorded. The data were then pooled for each aquarium and aquarium scores calculated by the formula:

$$Aquarium\ Score = \frac{\sum Individual\ Fish\ Score}{N}$$

N = Number of fish in the aquarium. Score domain: 0-4.

Immediately following the hemoglobin test, fish were killed by an overdose (ca. 0.3 g/l.) of the anesthetic tricaine methanesulfonate (MS-222). Total length and weight for each fish were measured and the condition index (K) was calculated by the formula below (Kneib and Stiven 1978). The results were averaged for each aquarium.

$$K = W/L^3$$

W: weight (g) x 100 to nearest 0.1 g

L: total length (cm) to nearest 1 mm

Histological method:

All fish were examined for external skin lesions. The number, severity and location of the lesions were recorded for each fish and the number totalled for each aquarium. The fish were then decapitated and their tail and internal organs were removed. The carcasses, including the body walls between the pectoral and anal fins, were fixed in 5% saline formaldehyde for ca. 24 hours, then washed in running water for one hour. The carcasses were cut in half (left and right) and the ventral areas, each ca. 0.8 cm^2, were retained. This area was chosen because of the low number of melanophores present in the skin, which facilitated counting of the goblet cells. The halves (20 per aquarium) were randomly distributed between the two stains ($AB_{1.0}$ or $AB_{2.5}$), but any two halves from a single fish were allotted different stains. After a 5 min. treatment in alcian blue, the halves were blotted dry ($AB_{1.0}$) or rinsed in running water for 5 min. ($AB_{2.5}$). They were immersed in a 1% periodic acid solution for 6 min., washed in running water for 5 min. and immersed in the Schiff's reagent solution for 3 min. The tissue specimen were then rinsed three times in 0.5% sodium metabisulfite solution, 20 min. per rinse, and washed in running tap water for 30 min. The attached, underlying muscle tissue which had prevented staining of the skin from the basal side was removed and the skin was dehydrated in a series of ethanol solutions of increasing concentrations (30 to 100% EtOH). The dehydrated tissue was cleared in three 15 min. changes of methyl salicylate and was permanently mounted on a slide for microscopic (600x) examination. Ten randomly selected, non-overlapping fields were examined. Within each field, goblet cells of each type were counted in a

0.01 mm^2 area and the diameters of three cells of each mucus type were measured. The results were expressed as the mean number/mm^2 and the average cell diameter for each aquarium.

Statistical treatment

As temperature and salinity did not differ between treatments, only their means and standard deviations are reported. Total organic carbon values of the treatment and control sediments are reported as % dry weight. Percent saturation of dissolved oxygen and pH were analyzed using analysis of covariance with sediment type as the main effect and time as a covariate. The DOC data were analyzed using analysis of variance. Further testing with the Tukey multiple comparison test differentiated between the daily values of the two treatments. The analyses of the oxygen saturation, pH and DOC were conducted only with the values obtained from the aquaria in operation throughout the experimental period.

A Kruskal-Wallis test was conducted on the Hb data. Regressions were performed on the condition indices of the control and treatment aquaria and the time series measurements were analyzed with the Tukey multiple comparison test. Regressions were performed on fish length and weight to determine which caused the changes in condition index. The diet of the fish (% body weight / day) was analyzed by factorial model to determine if there were differences between treatments. The lesion abundance and mortality data were analyzed by Kruskal-Wallis tests.

To ensure that the same overall goblet cell population was examined using the two different stains, paired t-tests were used to compare the density and diameter of the goblet cells counted in each stain, using data from individual fish. The proportion of each mucin type was arcsine transformed to satisfy the normality and homoscedasticity assumptions for all subsequent analyses. Regressions were performed on the goblet cell diameter by type (blue, red and mix), mean diameter and pH of the alcian blue. Tukey multiple comparison tests were performed on their diameter and on the goblet cell mucin type, by type and by pH of the alcian blue.

Results

General observations:

Shortly after introduction of the fish into the aquaria, some fish in both treatment and control aquaria jumped out. Thereafter, the aquaria were covered with a plastic sheet to prevent further losses. Particulates resuspended in the water and the coloration pattern of the mummichog prevented an accurate count. As the experiment progressed, a decrease in swimming activity, more evident in fish of treatment than control aquaria, resulted in lower turbidity. Counts done *a posteriori* showed that of the 20 fish that jumped out of the aquaria, 13 were from control aquaria. These 20 fish were discarded. Nine fish died during the course of the experiment. All deaths occurred in treatment aquaria and were distributed over the whole experimental period, from Days 3 to 13. There was a significant difference in survival at the 5% level between the treatments. Final counts are presented in Table 3.

Table 3. Fish counts and lesion data from the experimental aquaria.

Aquaria	Initial Number	Mortality	Day of Death	Final Number	Lesion Occurrence		
					Fin	Anal	Other
C-0	10	0		10	0	0	0
C-0	10	0		10	0	0	0
C-3	9	0		9	0	0	0
C-3	10	0		10	0	0	0
C-3	8	0		8	1	0	0
T-3	11	0		11	0	0	0
T-3	11	0		11	0	0	0
T-3	9	0		9	0	0	0
C-7	6	0		6	0	0	0
C-7	10	0		10	0	0	0
C-7	11	0		11	0	0	0
T-7	10	0		10	2	0	0
T-7	10	2	6,6	8	0	0	2
T-7	10	0		10	9	0	0
C-13	9	0		9	2	0	0
C-13	7	0		7	0	0	0
C-13	8	0		8	0	0	0
T-13	10	4	3, 4, 9, 10	6	8	4	0
T-13	7	2	7, 13	5	9	1	1
T-13	7	1	13	6	13	0	2

Gross epidermal lesions were rare until Day 7. Only one fish, from a control aquarium on Day 3, exhibited a minor fin erosion before that day. Overall, 54 lesions were noted. Fin erosions accounted for 74% of all lesions and ulcerations of the epidermis surrounding the anus for 13%. All remaining lesions (13%) consisted of epidermal abrasions and ulcerations of varying size, which sometimes penetrated the integument and in some fish exposed the internal organs. One such ulceration, on a fish (Total length = 91 mm) which died on Day 13, was estimated at 1.75 cm^2. Fish from treatment aquaria exhibited over 94% of the lesions, a significantly higher percentage ($p < 0.05$) than that of control aquaria.

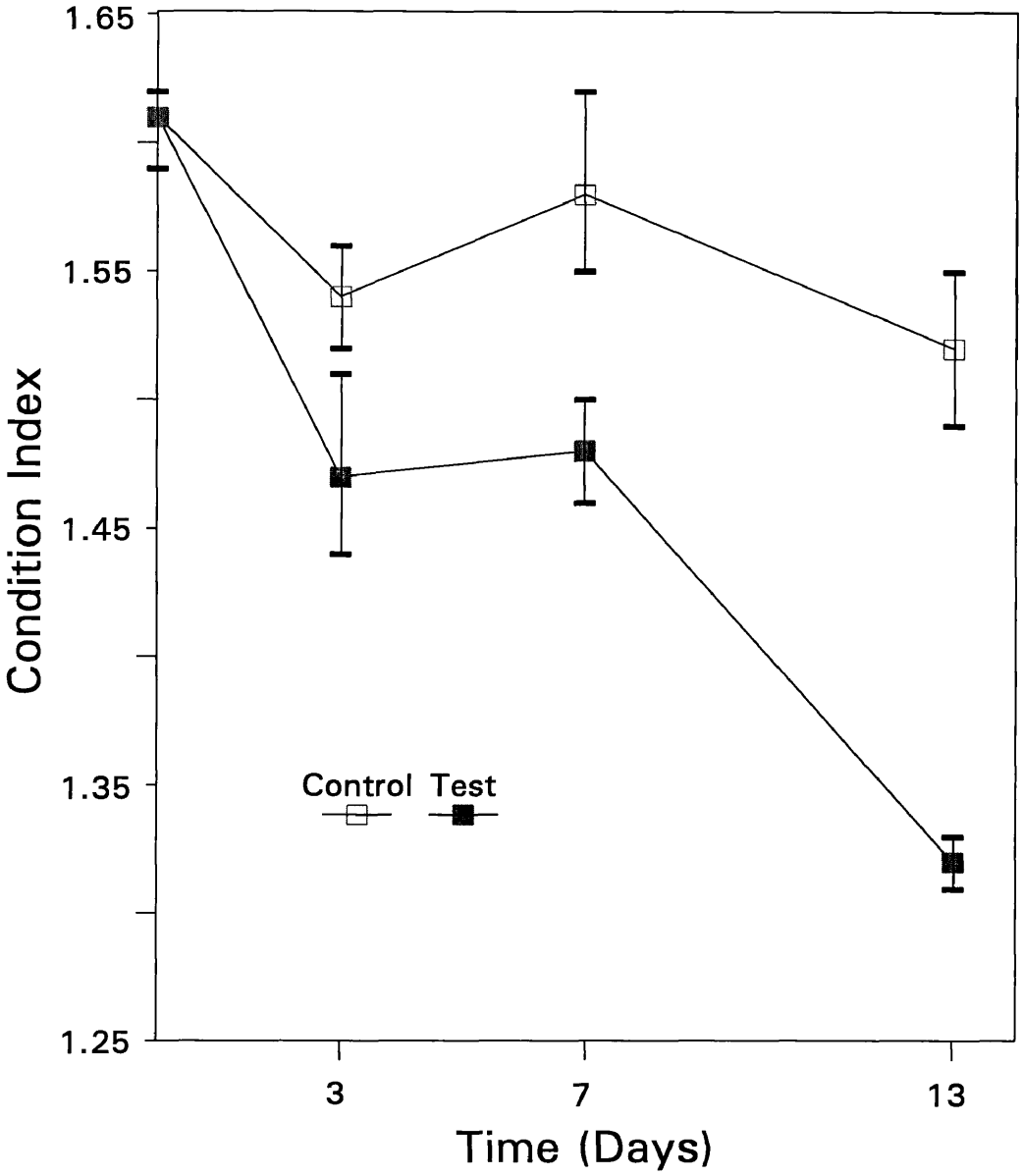
The condition index (CI) of control fish averaged 1.56 ($\pm$ 0.04) and did not change during the experiment. The CI of treated fish decreased over time ($p < 0.01$) and was significantly different from that of control fish (Fig. 5). The regression of the treated fish CI against time was:

$$CI = 1.55 - 0.016 \times day$$

These changes were due to a weight loss. The total length of treated fish did not vary significantly with time nor did it differ from the length of control fish. There was, however, a significant decrease in weight of treated fish ($p < 0.01$) even though all aquaria received the same amount of food. The regression of treated fish weight against time was:

$$Weight = 10.17 - 0.15 \times day$$

Figure 5. Condition index of mummichog exposed to control and treatment (creosote-contaminated) sediments. Mean, standard deviation and Tukey multiple comparison test results (n=3, except for C-0 where n=2). Aquaria underlined by the same line are not significantly different ($p < 0.05$).



C-0 C-7 C-3 C-13 T-7 T-3 T-13

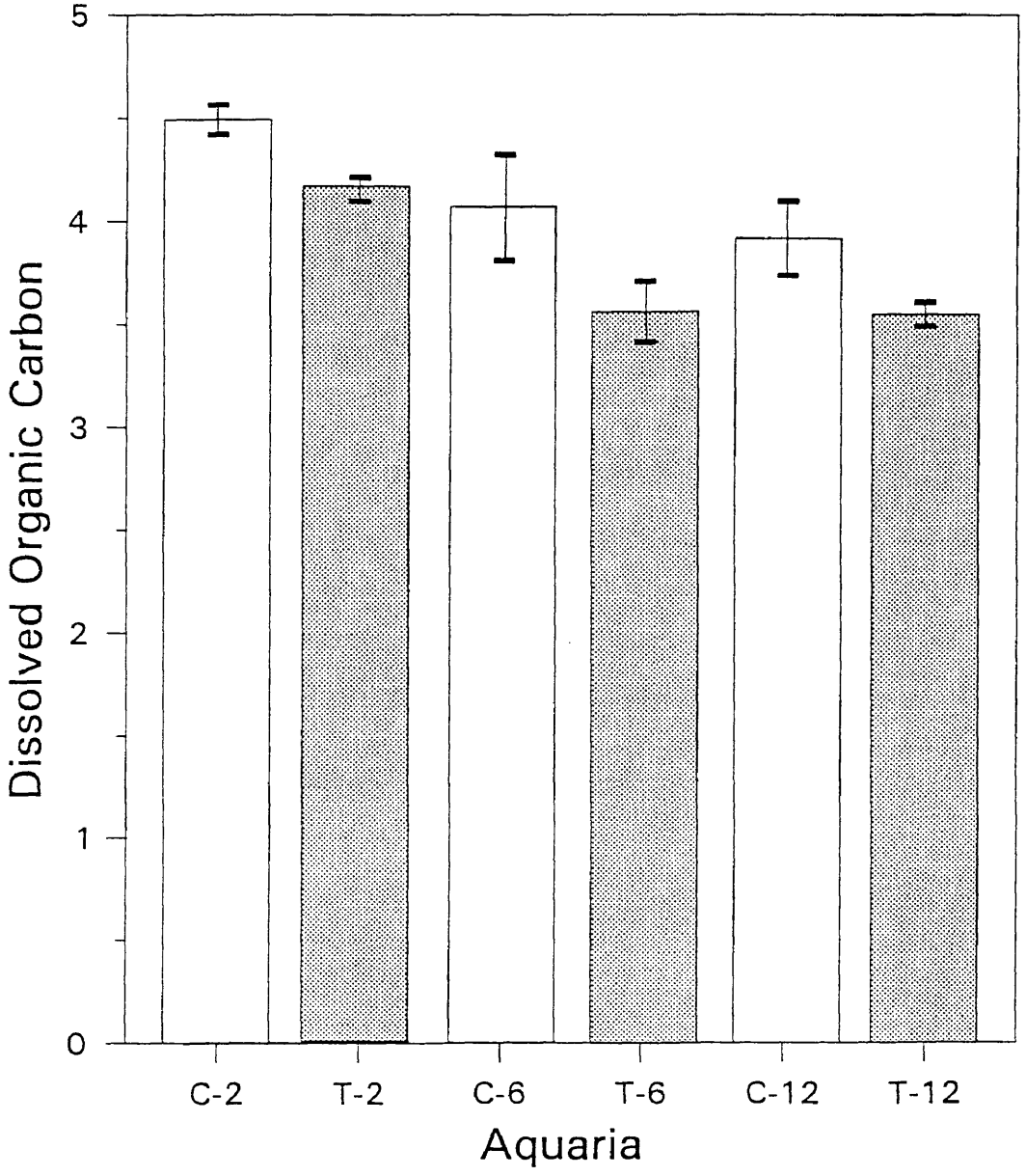
Tukey Multiple Comparison Test

Water and Sediment Quality:

The water temperature and salinity were stable and did not differ between aquaria with mean values (± standard deviation) of 27.9 °C (± 0.7) and 22.2 ppt (± 0.4) respectively. The pH did not differ significantly ($p < 0.93$) between treatments (mean: 7.8 ± 0.05). Dissolved oxygen levels (DO) did not differ between treatments ($p < 0.22$) and averaged 80% of saturation (± 5). A single incidence of low DO was observed, in a control aquarium at Day 3 (49% saturation). The fish in that aquarium were not processed until Day 13. Dissolved organic carbon (DOC) was significantly higher in the control aquaria than in the treatment aquaria ($p < 0.001$) and decreased over time ($p < 0.001$) in both treatments (Fig. 6). The total organic carbon of the control sediment was 5.5% on a dry weight basis. That of the treatment sediment was 8.8%.

Analysis of effluent from treatment aquaria showed a wide variety of aromatic compounds present in the water column, either dissolved in the water or sorbed to suspended sediment (Table 4). The mean total resolved aromatic compound concentration for treatment aquaria at Day 2 was 125 μg/l (± 44.9). That concentration decreased by a factor of five by Days 6 (23.1 μg/l ± 5.3) and 12 (17.0 μg/l ± 5.4). The control aquarium total aromatic compound concentration was 3.7 μg/l (Fig. 7).

Figure 6. Dissolved organic carbon (mg/l) in the aquarium effluents. Mean, standard deviation and Tukey multiple comparison test results (n=3). Aquaria underlined by the same line are not significantly different ($p < 0.05$).



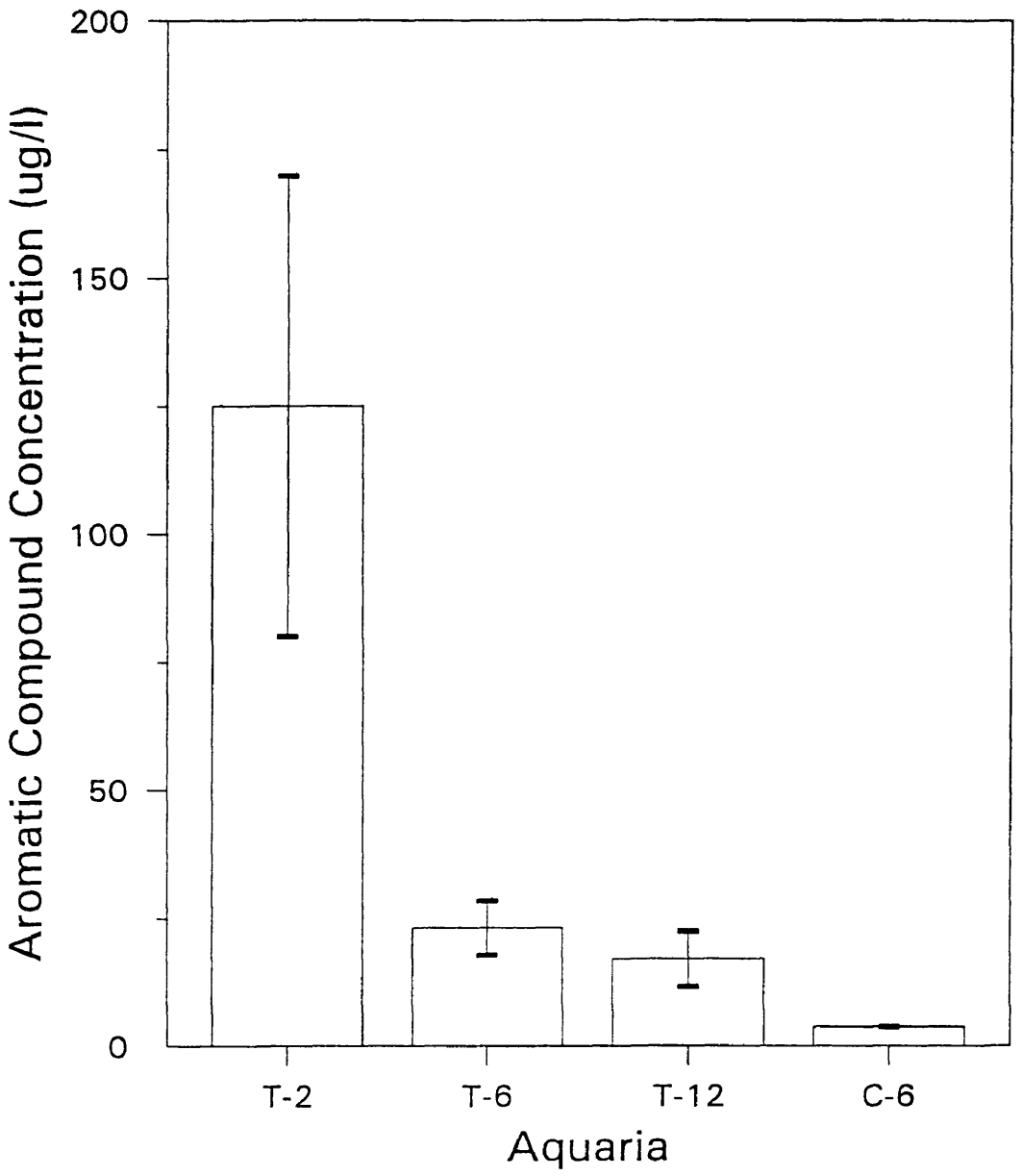
C-2 T-2 C-6 C-12 T-6 T-12

Tukey Multiple Comparison Test

Table 4. List of the polycyclic aromatic hydrocarbons and heterocyclic analogues identified in the aquarium effluents by GC/MS. Their mean concentrations (µg/l) and standard deviations from the Day 2 samples are included (n=9).

Aromatic Compound	Mean Conc.	Std. Dev.
Naphthalene	0.30	0.43
2-Methyl-naphthalene	0.94	0.95
1-Methyl-naphthalene	1.97	0.75
C2H5-naphthalene	3.10	1.89
Acenaphthene	11.20	4.09
Dibenzofuran	4.46	1.96
Fluorene	6.68	2.70
Phenanthrene	26.00	11.90
Anthracene	6.83	7.19
Carbazole	0.47	0.26
Cyclopenta(def)phenanthrene, 4H	3.22	1.33
Fluoranthene	16.90	6.73
Pyrene	10.20	4.22
Phenylnaphthalene	2.20	0.77
Benzo(a)fluorene	3.08	1.13
Benzo(b)fluorene	3.25	1.35
Benzo(a)anthracene	3.81	1.57
Chrysene	3.22	1.70
Benzo(k)fluoranthene	4.31	1.76
Benzo(j)fluoranthene	1.37	0.81
Benzo(b)fluoranthene	4.20	2.03
Benzo(a)pyrene	4.71	4.60
Perylene	1.14	0.98
Indeno(1,2,3-cd)pyrene	0.47	0.31
Dibenz(a,h)anthracene	0.93	0.76
Total	125	44.9

Figure 7. Total resolved aromatic compound concentrations (μg / l) (± standard deviation) in aquarium effluents (± standard deviation). Each bar represents the sum of the concentrations of the compounds (T-2: n=9; T-6: n=6; T-12: n=3; C-6: n=1).


200
150
100
50
0
Aromatic Compound Concentration (ug/l)
T-2
T-6
T-12
C-6
Aquaria

Hemoglobin assay:

The fish assayed on Day 0 showed a low but detectable level of free Hb in their mucus (Fig. 8). 40% of the fish had a moderate response, 15% a low response and 45% showed only trace levels of Hb. On Day 3, control fish exhibited a higher Hb level than on day 0, with 8% (2 fish) having a high Hb response. By Day 7, control fish exhibited neither a high nor a trace response. Most exhibited a low response and the remainder, a moderate response. On Day 13, 8% (2 fish) of the control fish again exhibited a high Hb response and 65% (15 fish) a moderate one. The remainder still exhibited a low response. Treated fish on Day 3 had a higher response than control fish at any time with 72% of the fish exhibiting a high response. On Days 7 and 13, all of the treated fish had high levels of Hb associated with the epidermal mucus. Overall, the Hb levels in fish from the treatment aquaria were significantly higher than those in fish from the control aquaria ($p < 0.01$).

Epidermal parameters:

Plate 1 illustrates the different mucin stains. At an alcian blue pH of 1.0 (Plate 1a), sulfated mucins stained blue, non-sulfated mucins stained red and mixed-sulfated mucins stained purple. At an alcian blue pH of 2.5 (Plate 1b), acid mucins stained blue, neutral mucins stained red and mixed-acid mucins stained purple. At low magnification (60x), the skin samples from fish that had not been exposed to sediment (C-0) showed a high density of goblet cells (Plate 2a, 2b). The scales are not visible, the epidermal surface is smooth and fat vacuoles sometimes are visible on

Figure 8. Hemoglobin response in the epidermal mucus of the mummichog, *Fundulus heteroclitus*, exposed to control and treatment (creosote-contaminated) sediment (n=3, except for C-0 where n=2).

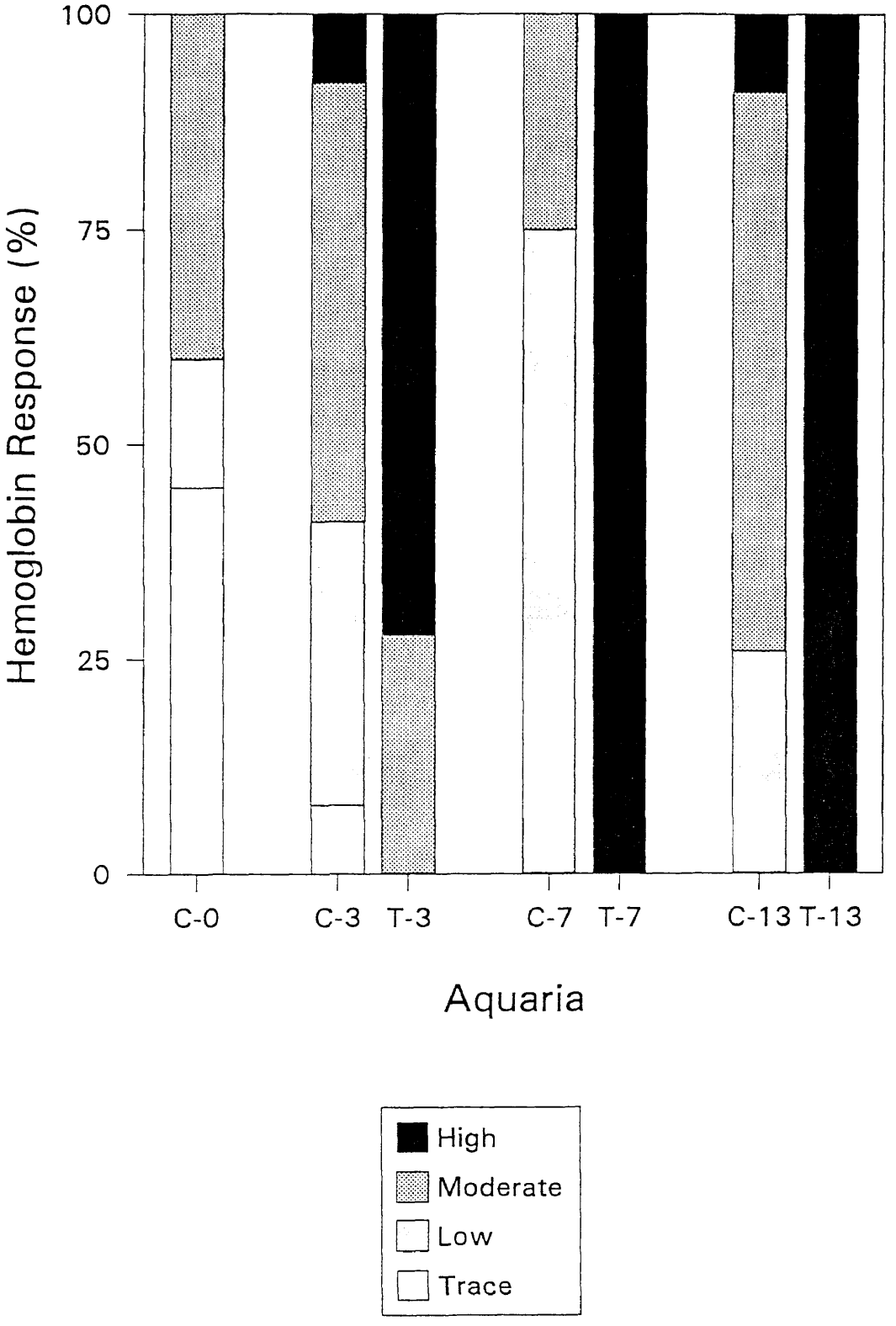

100
75
50
25
0
Hemoglobin Response (%)
C-0
C-3
T-3
C-7
T-7
C-13
T-13
Aquaria
High
Moderate
Low
Trace

Plate 1. Epidermal goblet cells of a mummichog, *Fundulus heteroclitus,* prior to exposure to sediment (570x).
a: alcian blue (pH=1.0) / PAS. b: alcian blue (pH=2.5) / PAS.
Bar = 20μm.

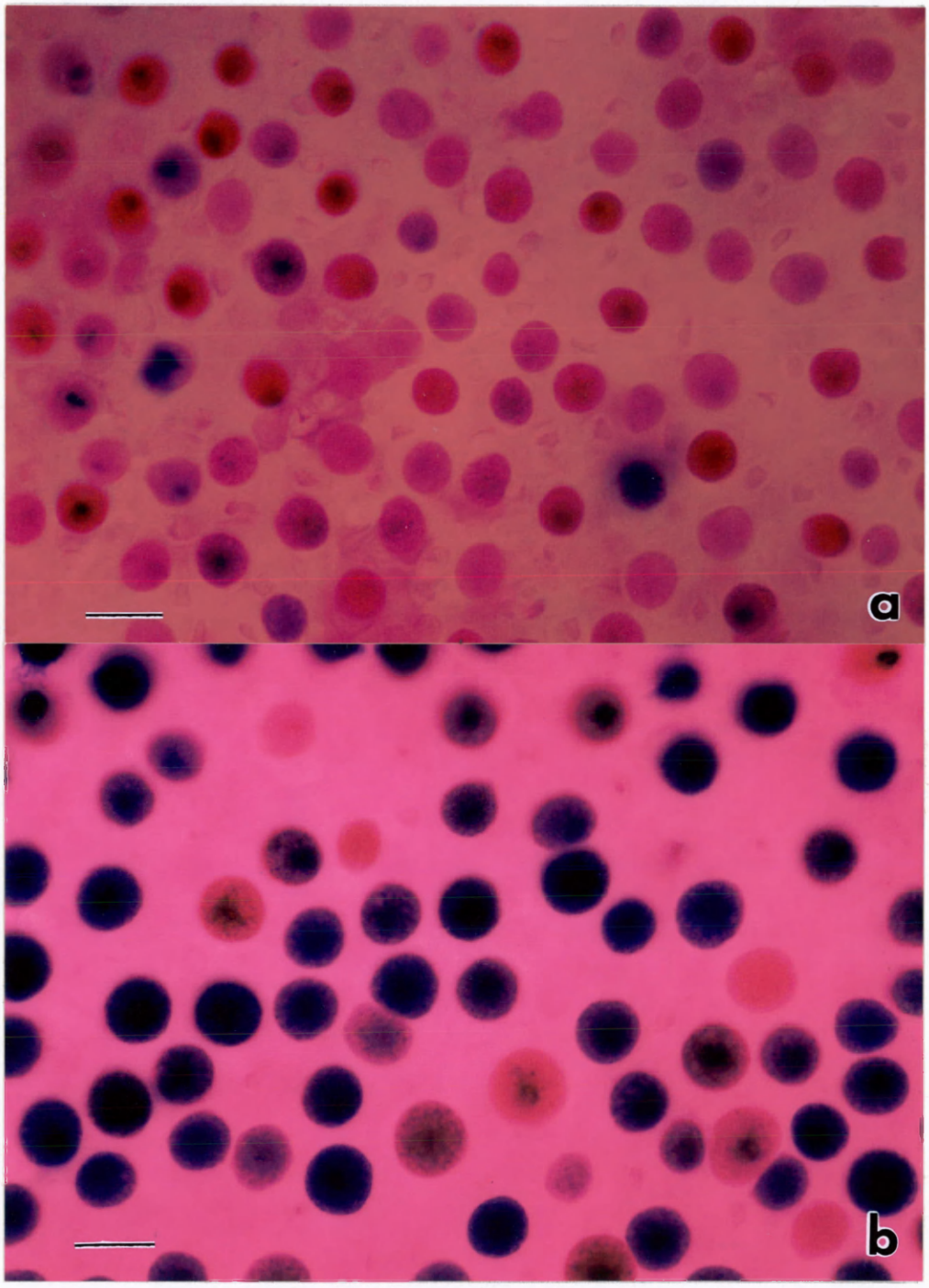
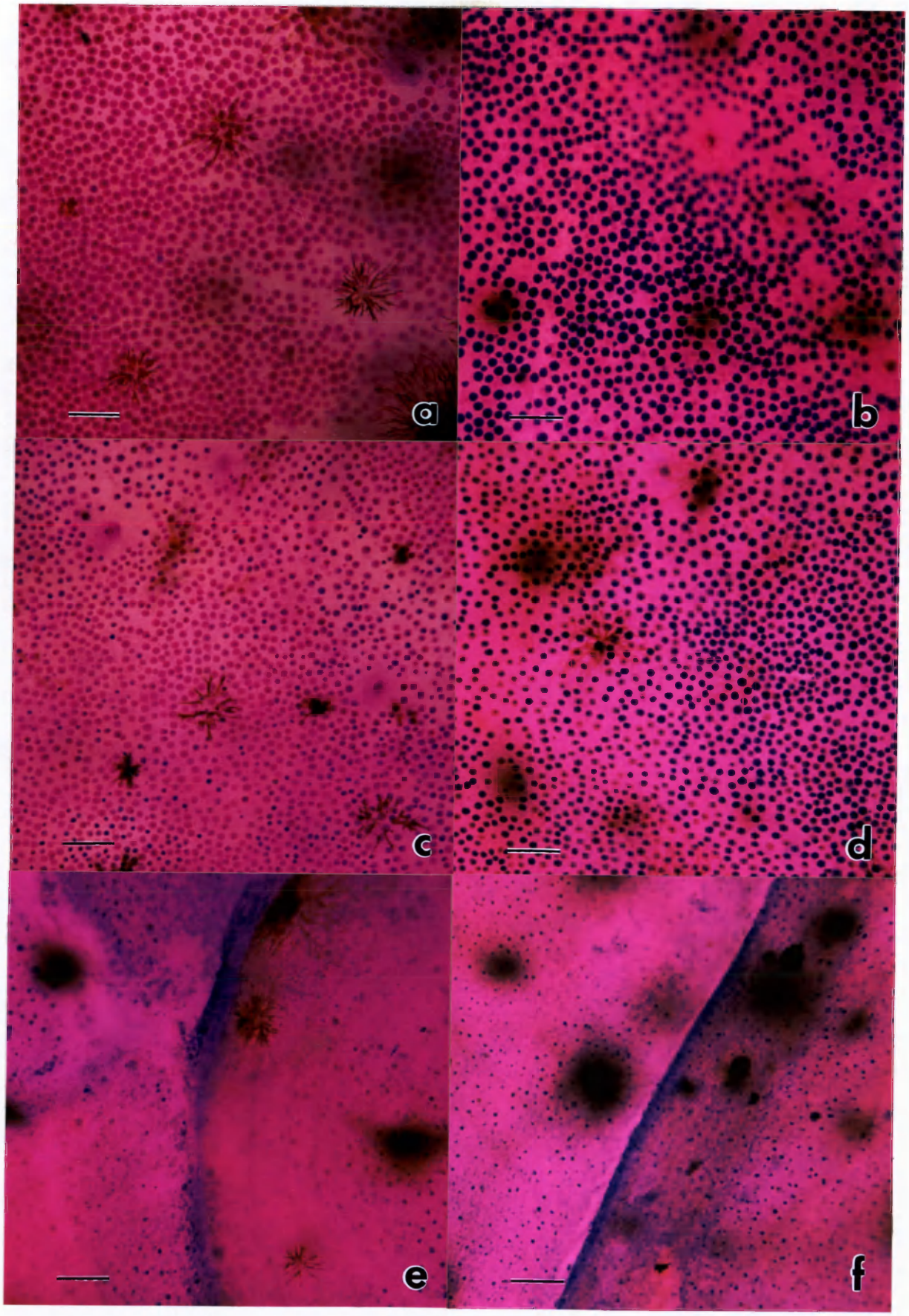

a
b

Plate 2. Epidermal goblet cells of the epidermis of a mummichog, *Fundulus heteroclitus*, prior to and after a 13 day exposure to control or treatment (creosote-contaminated) sediment (90x).

a: alcian blue (pH=1.0) / PAS	prior to exposure
b: alcian blue (pH=2.5) / PAS	prior to exposure
c: alcian blue (pH=1.0) / PAS	control sediment
d: alcian blue (pH=2.5) / PAS	control sediment
e: alcian blue (pH=1.0) / PAS	treatment sediment
f: alcian blue (pH=2.5) / PAS	treatment sediment

Bar = 100μm.


a
b
c
d
e
f

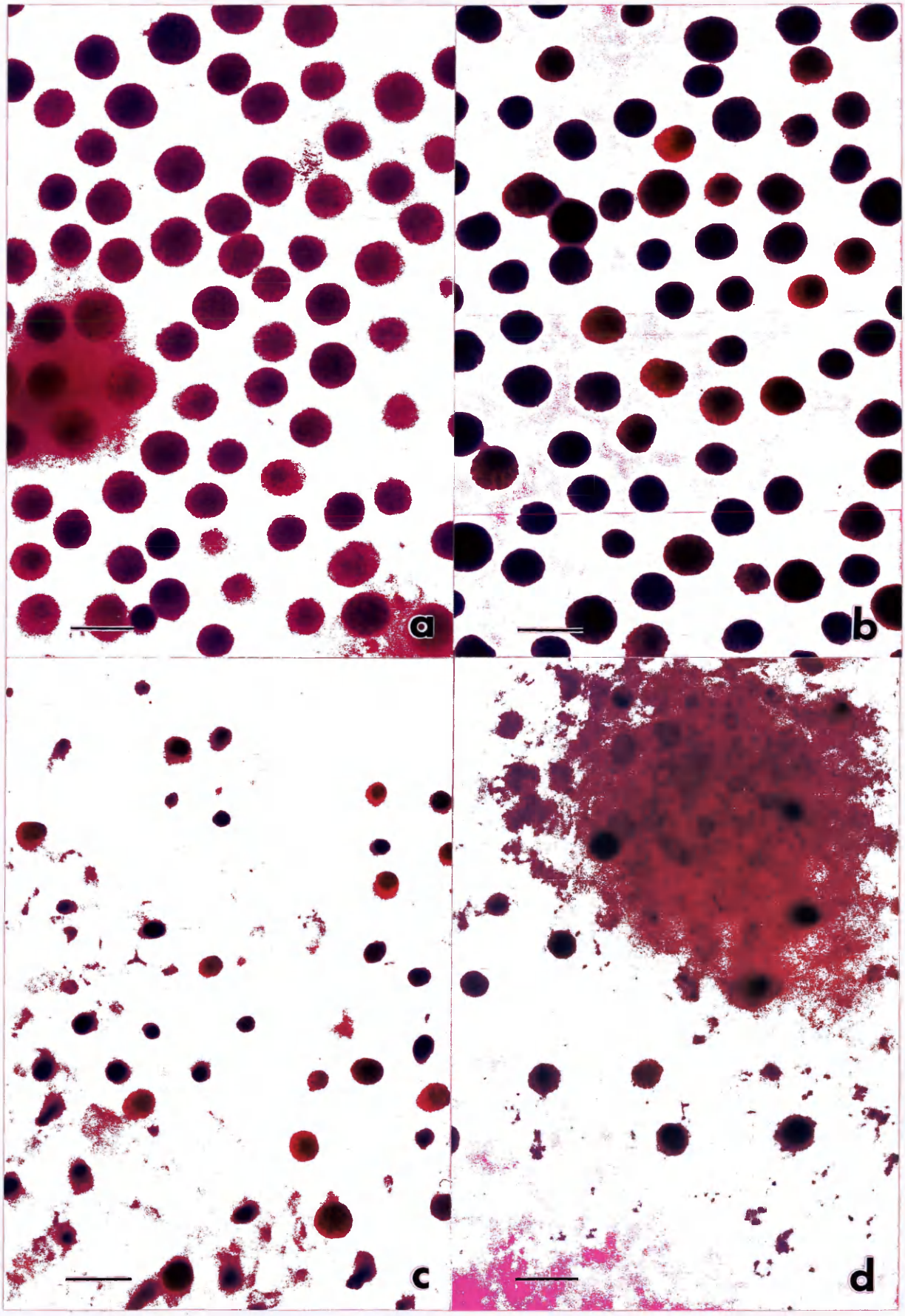
the edge of the tissue. After a 13-day exposure to control sediment (C-13), the skin samples still showed a high density of goblet cells (Plate 2c, 2d), and did not appear greatly different from the C-0 skin samples. The 13-day exposure to treatment sediment (T-13) greatly altered the appearance of the skin of treated fish (Plate 2e, 2f). The epidermal goblet cells appeared smaller and less densely distributed. The scales, which were not visible in any other sample, were conspicuous and salient. No fat vacuoles were visible in any of the T-13 skin samples. At higher magnification (600x), there was no obvious difference between the C-0 and C-13 samples (Plates 1a, 1b and 3a, 3b). The goblet cells of these skin samples appeared to have the same overall diameter and density. The change in diameter and density of the epidermal goblet cells in fish exposed to treatment sediment for 13 days was obvious at 600x (Plate 3c, 3d). The diameter of the goblet cells from the T-13 fish was approximately half that of the C-0 and C-13 fish. The density of the cells also appeared greatly reduced. Further, more examples of small lesions and epithelial cell proliferation in areas devoid of goblet cells were seen in the T-13 samples than in the C-13 samples.

Goblet cell population:

The goblet cells were counted and their diameters measured in two different stains. There were no significant differences between the density of goblet cells per fish in each stain ($p < 0.25$) or between their mean diameter ($p < 0.79$).

Plate 3. Epidermal goblet cells of the epidermis of a mummichog, *Fundulus heteroclitus*, after a 13 day exposure to control or treatment (creosote-contaminated) sediment (570x).

a: alcian blue (pH=1.0) / PAS control sediment
b: alcian blue (pH=2.5) / PAS control sediment
c: alcian blue (pH=1.0) / PAS treatment sediment
d: alcian blue (pH=2.5) / PAS treatment sediment
Bar = 20μm.


a
b
c
d

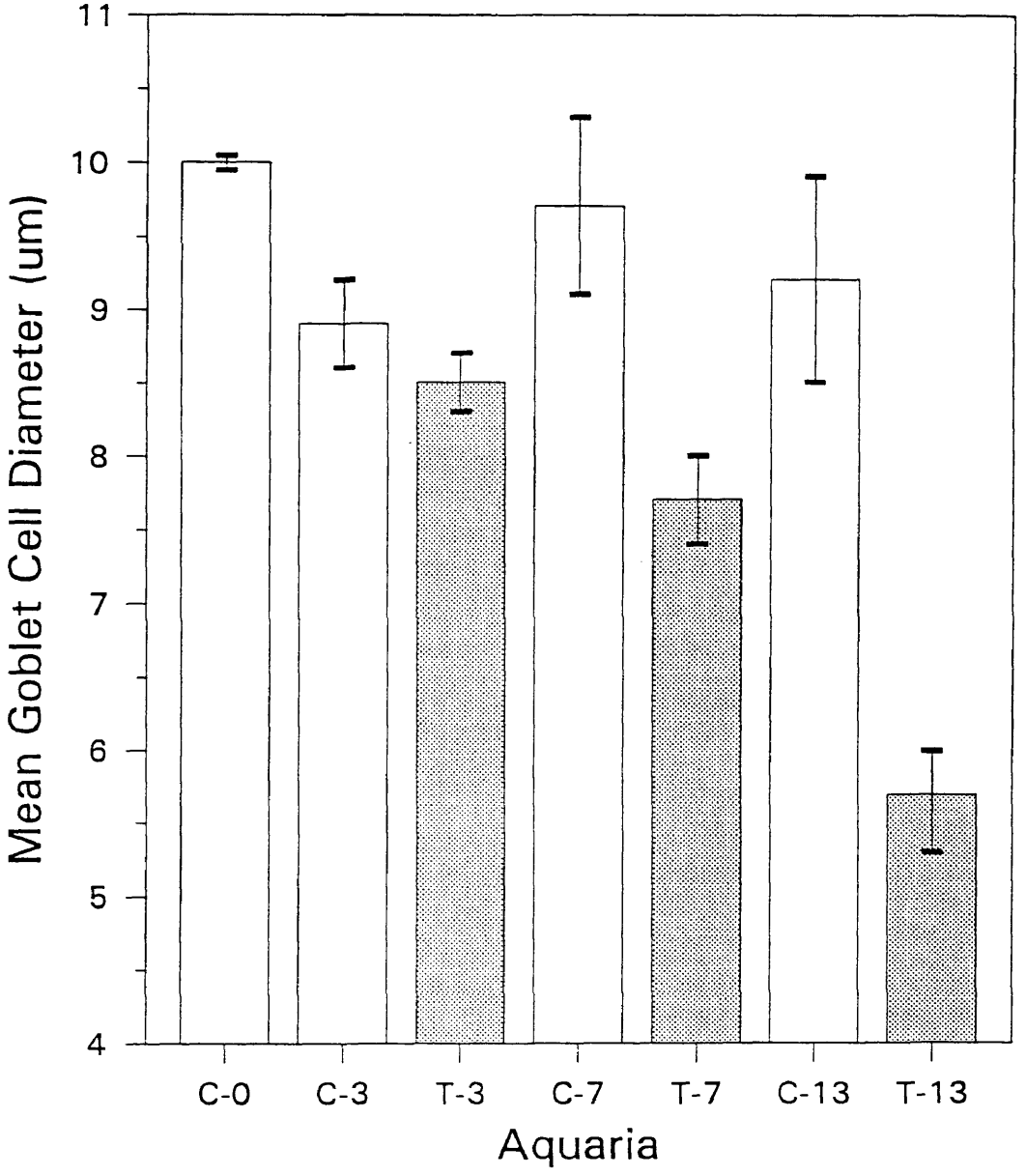
Goblet cell diameter:

At the start of the experiment (Day 0), the mean epidermal goblet cell diameter (of a fish per aquarium) was 10 μm (± 0.1) (Fig. 9). On Day 3, that diameter had diminished in both the control (C-3) and treated fish (T-3), to 8.9 μm (± 0.4) and 8.5 μm (± 0.2), respectively. By Day 7, the goblet cell diameter of control fish (C-7) had recovered (9.7 μm ± 0.6) and remained high on Day 13 (9.2 μm ± 0.8). A regression analysis on the diameter of the goblet cells from control fish showed that the slope was not significantly different from 0. The cell diameters of treated fish from the T-7 and T-13 aquaria did not return to C-0 levels, as did those of control fish. The mean cell diameters of T-7 and T-13 fish were 7.7 μm (± 0.3) and 6.0 μm (± 0.4), respectively. Regression analyses on treated fish goblet cell diameters showed that the slopes were significantly different from 0 ($p < 0.01$) and did not differ between mucin types. The decrease in the diameter of treated fish goblet cells was as follows:

$$\text{Diameter} = 9.7\ \mu\text{m} - 0.31 \times \text{Day}$$

Tukey multiple comparison test results on goblet cell diameter (Fig. 9) indicated that the goblet cell diameter of C-3 fish is significantly ($p < 0.05$) smaller than that of C-0 fish but not significantly different from those of C-7 and C-13 fish. The cell diameter of T-3 fish is not different from that of C-3, C-13 and T-7 fish. The cell diameter of T-13 fish is significantly smaller than those of all other fish.

Figure 9. Epidermal goblet cell diameter in mummichog, *Fundulus heteroclitus*, exposed to control and treatment (creosote-contaminated) sediment. Mean, standard deviation and Tukey multiple comparison test results (n=3, except for C-0 where n=2). Aquaria underlined by the same line are not significantly different ($p < 0.05$).



C-0 C-7 C-13 C-3 T-3 T-7 T-13

Tukey Multiple Comparison Test

Goblet cell density:

On Day 0, the mean density (± standard deviation) of epidermal goblet cells (of a fish per aquarium) was 3148 / mm^2 (± 182). This density stayed constant, both in control and treated fish through Day 7 (Fig. 10). C-13 fish showed no significant change in goblet cell density. T-13 fish goblet cell density decreased significantly ($p < 0.05$) to a third of other values (933 ± 94 / mm^2).

Mucin type proportions:

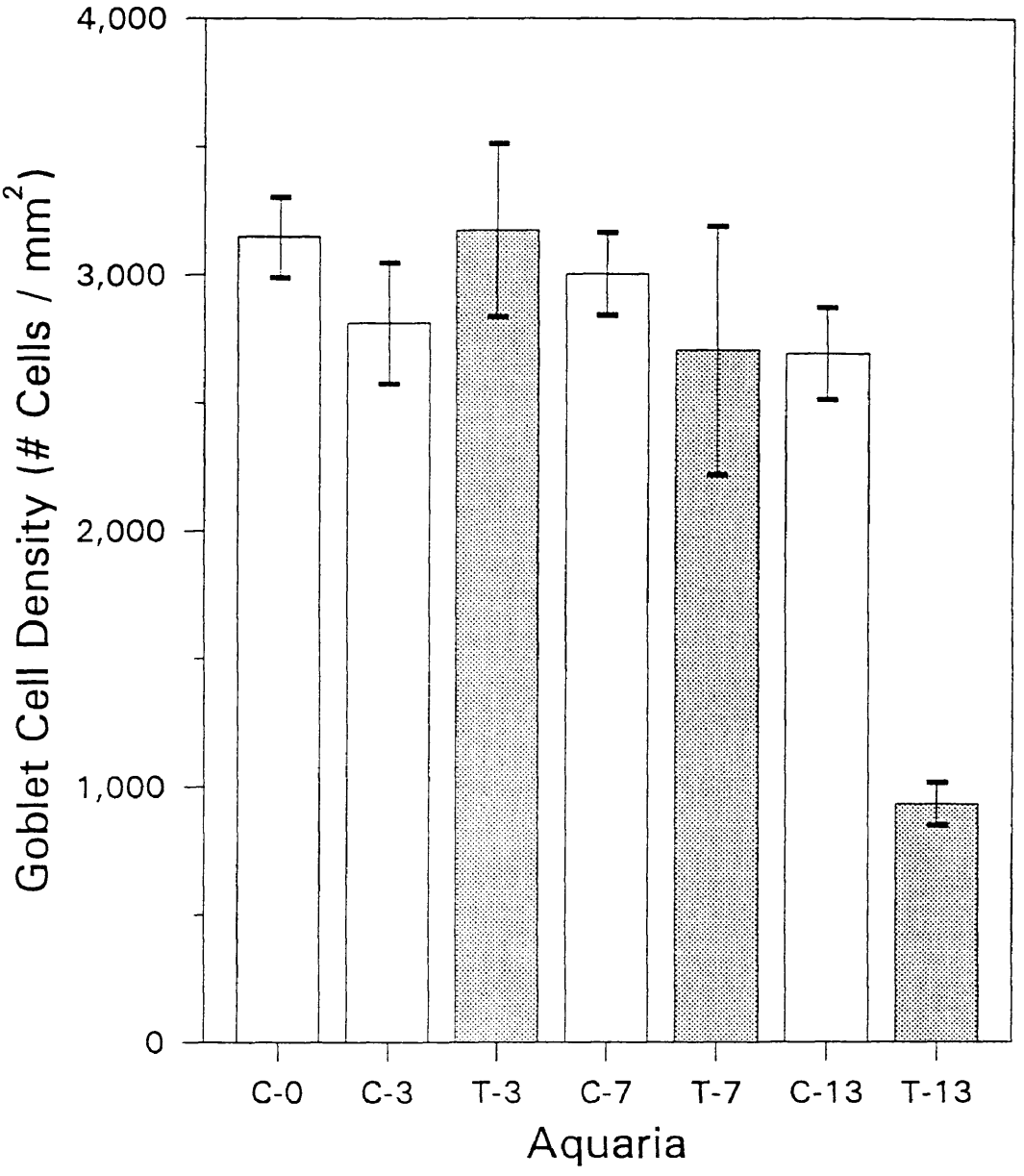
Sulfated mucins:

Goblet cells containing sulfated mucins constituted 4.9% (± 0.7) of the C-0 fish goblet cells (Fig. 11). On Day 3, that percentage had dropped in both control (C-3) and treated fish (T-3), to 0.8% (± 0.9) and 1.4% (± 0.7) respectively. Only 0.5% (± 0.3) of the C-7 fish goblet cells contained sulfated mucins, whereas the T-7 fish percentages exceeded the C-0 fish value, with 9.6% (± 2.7). On Day 13, the control fish values returned to C-0 levels, but with a greater variability (4.4% ± 4.4). The T-13 fish values continued an upward trend, with 16.2% (± 11.5) of the cells containing sulfated mucins resulting in a significant difference ($p < 0.05$) between the values of T-13 and the control fish C-3 and C-7.

Non-sulfated mucins:

The percentage of goblet cells containing non-sulfated mucins exhibited a trend opposite to that of the sulfated mucins (Fig. 12). From 58.2% (± 4.8) at Day 0,

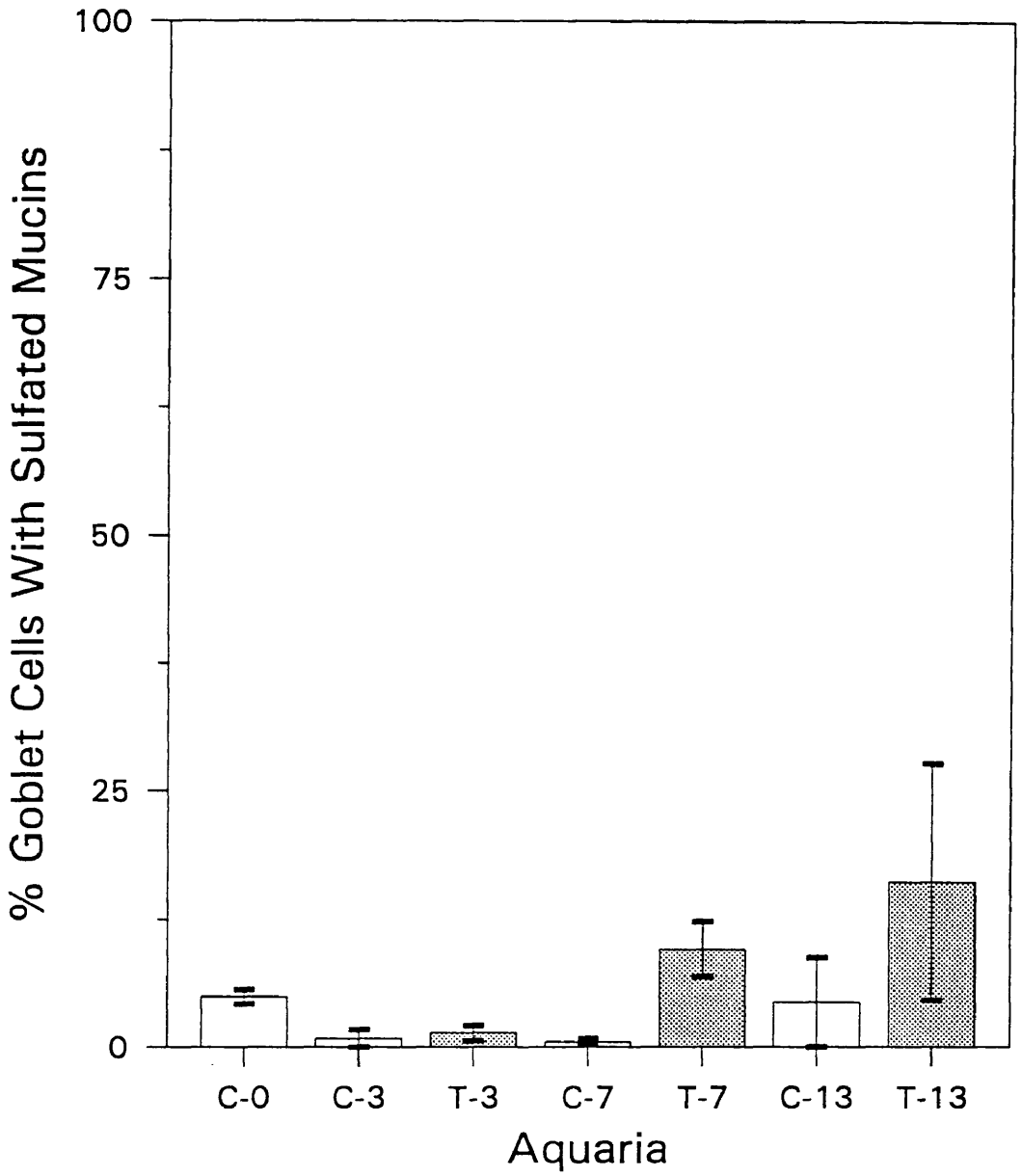
Figure 10. Epidermal goblet cell density in mummichog, *Fundulus heteroclitus*, exposed to control and treatment (creosote-contaminated) sediment. Mean, standard deviation and Tukey multiple comparison test results (n=3, except for C-0 where n=2). Aquaria underlined by the same line are not significantly different ($p < 0.05$).



T-3 C-0 C-7 C-3 T-7 C-13 T-13

Tukey Multiple Comparison Test

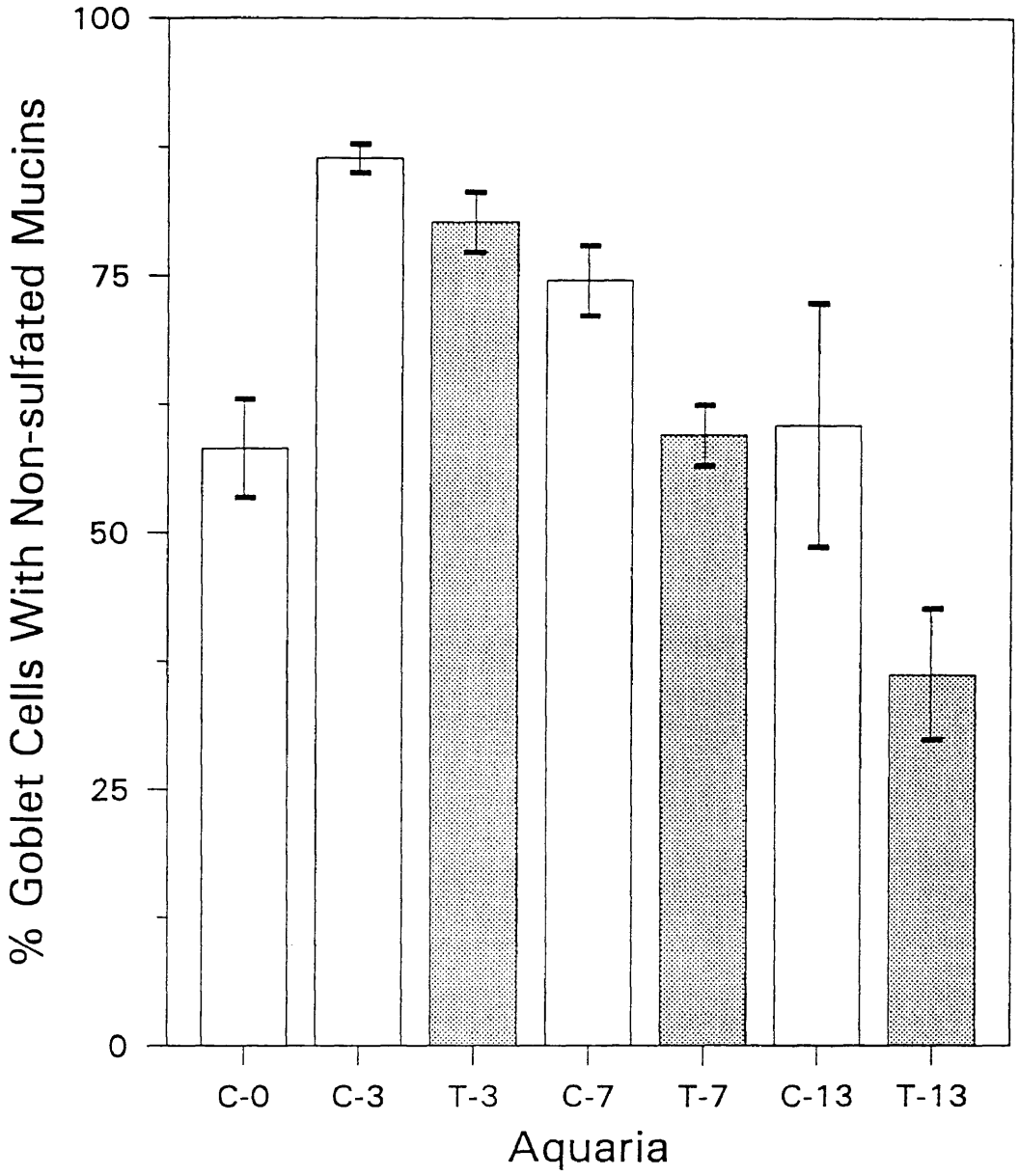
Figure 11. Percentage of epidermal goblet cells containing sulfated mucins in mummichog, *Fundulus heteroclitus*, exposed to control and treatment (creosote-contaminated) sediment. Mean, standard deviation and Tukey multiple comparison test results (n=3, except for C-0 where n=2). Aquaria underlined by the same line are not significantly different ($p < 0.05$).



C-7 C-3 T-3 C-13 C-0 T-7 T-13

Tukey Multiple Comparison Test

Figure 12. Percentage of epidermal goblet cells containing non-sulfated mucins in mummichog, *Fundulus heteroclitus*, exposed to control and treatment (creosote-contaminated) sediment. Mean, standard deviation and Tukey multiple comparison test results (n=3, except for C-0 where n=2). Aquaria underlined by the same line are not significantly different ($p < 0.05$).



C-3 T-3 C-7 C-13 C-0 T-7 T-13 C-0

Tukey Multiple Comparison Test

their percentages increased to 86.4% (± 1.4) and 80.2% (± 2.9) for C-3 and T-3 fish, respectively. The percentages in C-13 (60.4% ± 11.9) and T-7 fish (59.5% ± 3.0) were similar to C-0 values. The T-13 values (36.3% ± 6.4) were lower than those of C-0. The Tukey multiple comparison test ($p < 0.05$) indicated that C-3 percentages were significantly higher than those of all fish except C-7 and T-3 fish. C-0 percentages were not different from those of any other aquarium. T-13 percentages were significantly lower than those of all but C-0 fish. T-3 percentages were also significantly higher than T-7 percentages.

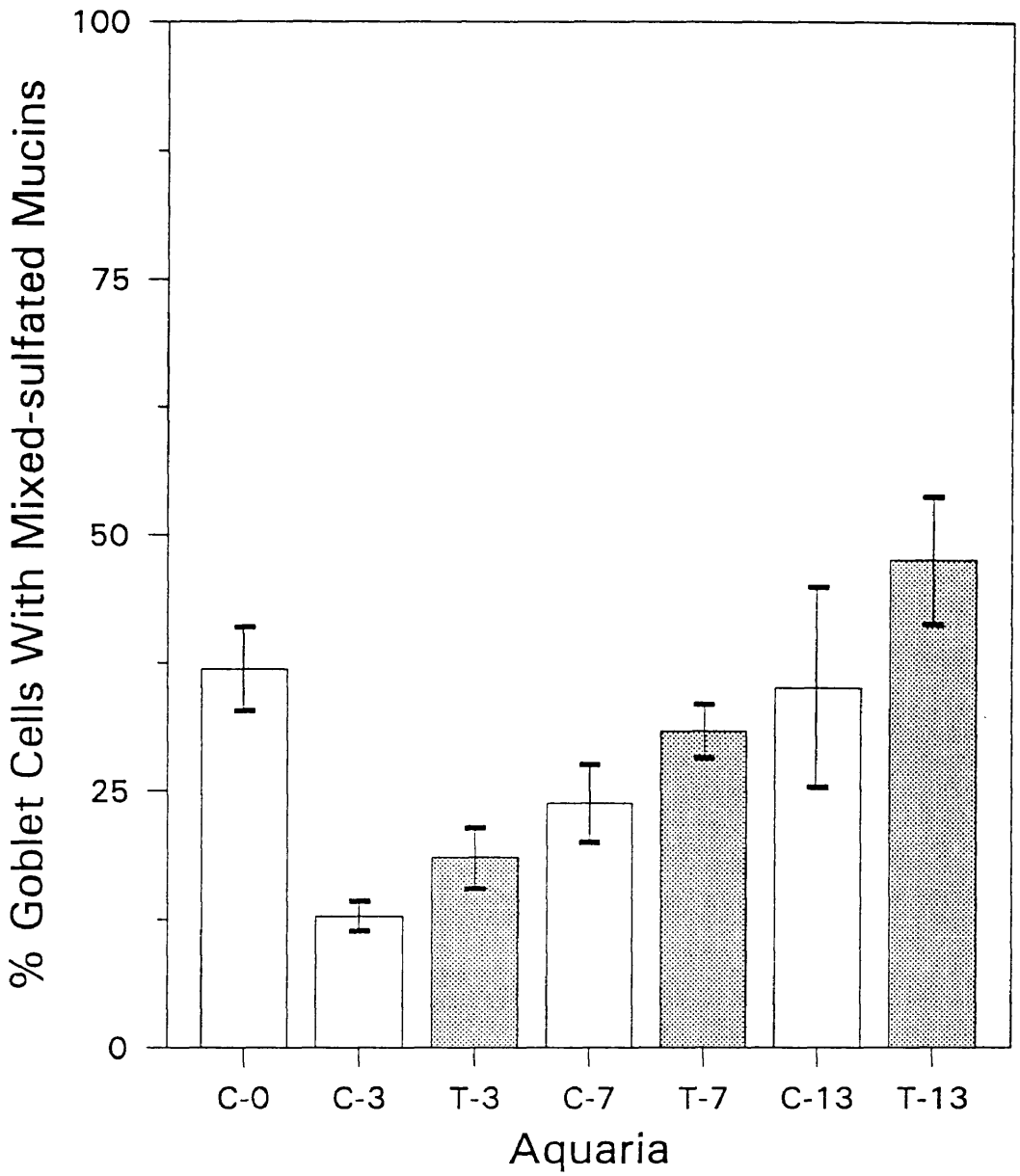
Mixed-sulfated mucins:

The percentages of goblet cells containing mixed-sulfated mucins changed in concert with those of cells containing sulfated mucins (Fig. 13). Starting at 36.9% (± 4.1), they decreased to 12.8% (± 1.4) for C-3 fish and 18.5% (± 2.9) for T-3 fish. The percentages then increased to reach C-0 levels for C-13 (35.1% ± 9.8) and T-7 fish (30.9% ± 2.6). T-13 percentages (47.5% ± 6.2) attained higher values than those of C-0. T-13 fish values were significantly higher ($p < 0.05$) than those of C-3, C-7 and T-3 fish. C-3 values were significantly lower than those of C-0.

Acid mucins:

Goblet cells containing acid mucins constituted 68.2% (± 5.4) of the C-0 fish goblet cells (Fig. 14). The percentages of these cells decreased from their Day 0 levels to 29% (± 14.8) for C-3 fish and 36.9% (± 10.5) for T-3 fish. These values

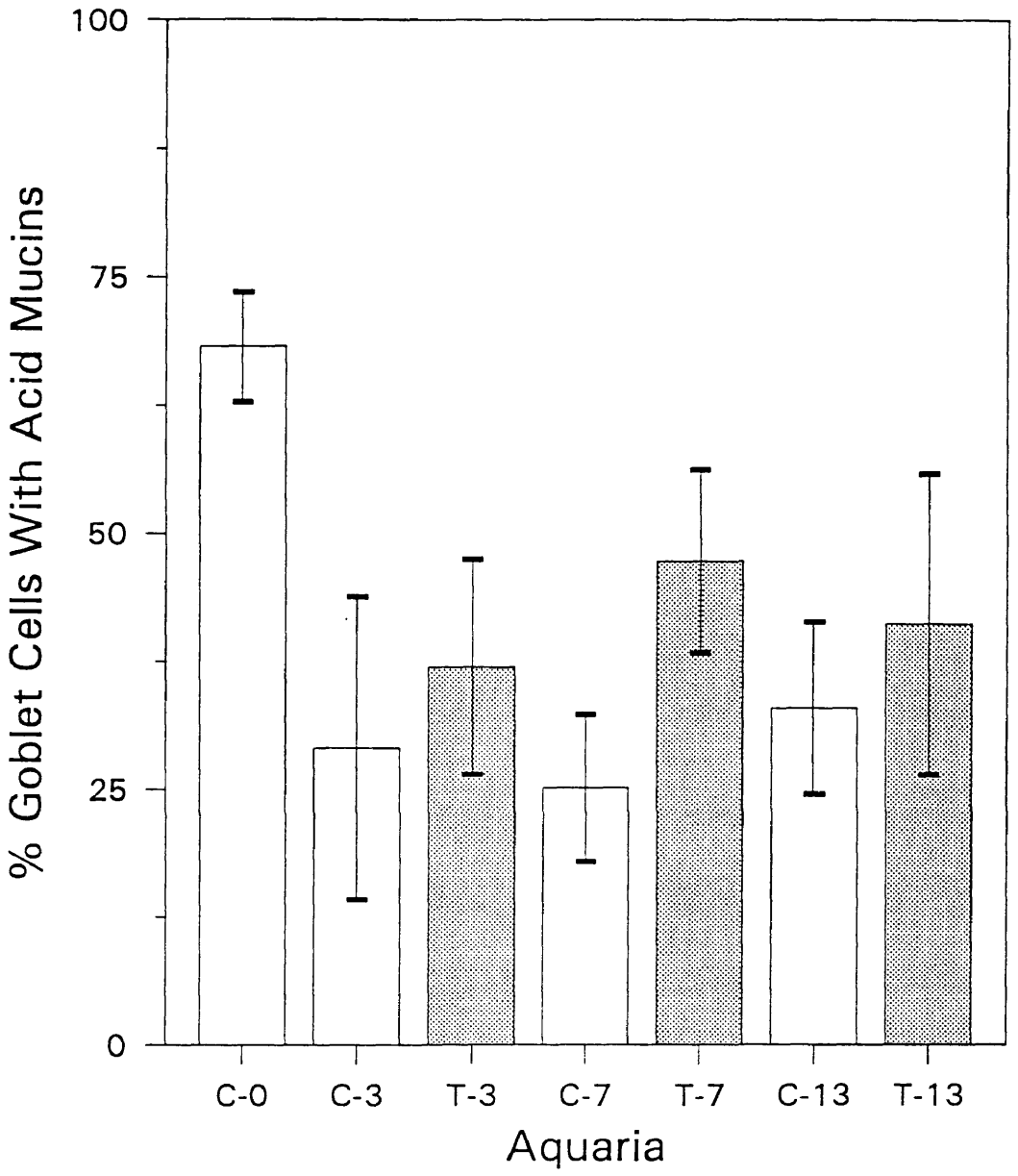
Figure 13. Percentage of epidermal goblet cells containing mixed-sulfated mucins in mummichog, *Fundulus heteroclitus*, exposed to control and treatment (creosote-contaminated) sediment. Mean, standard deviation and Tukey multiple comparison test results (n=3, except for C-0 where n=2). Aquaria underlined by the same line are not significantly different ($p < 0.05$).



T-13 C-0 C-13 T-7 C-7 T-3 C-3

Tukey Multiple Comparison Test

Figure 14. Percentage of epidermal goblet cells containing acid mucins in mummichog, *Fundulus heteroclitus*, exposed to control and treatment (creosote-contaminated) sediment. Mean, standard deviation and Tukey multiple comparison test results (n=3, except for C-0 where n=2). Aquaria underlined by the same line are not significantly different ($p < 0.05$).



C-0 T-7 T-13 T-3 C-13 C-3 C-7

Tukey Multiple Comparison Test

stayed constant throughout the rest of the experimental period, the treated fish maintaining slightly higher mean percentages than the control fish. The Tukey multiple comparison test indicated only one difference, between C-0 and C-7 fish. The C-7 percentage of acid mucins (25.1% ± 7.2) was significantly lower ($p < 0.05$) than the C-0 values.

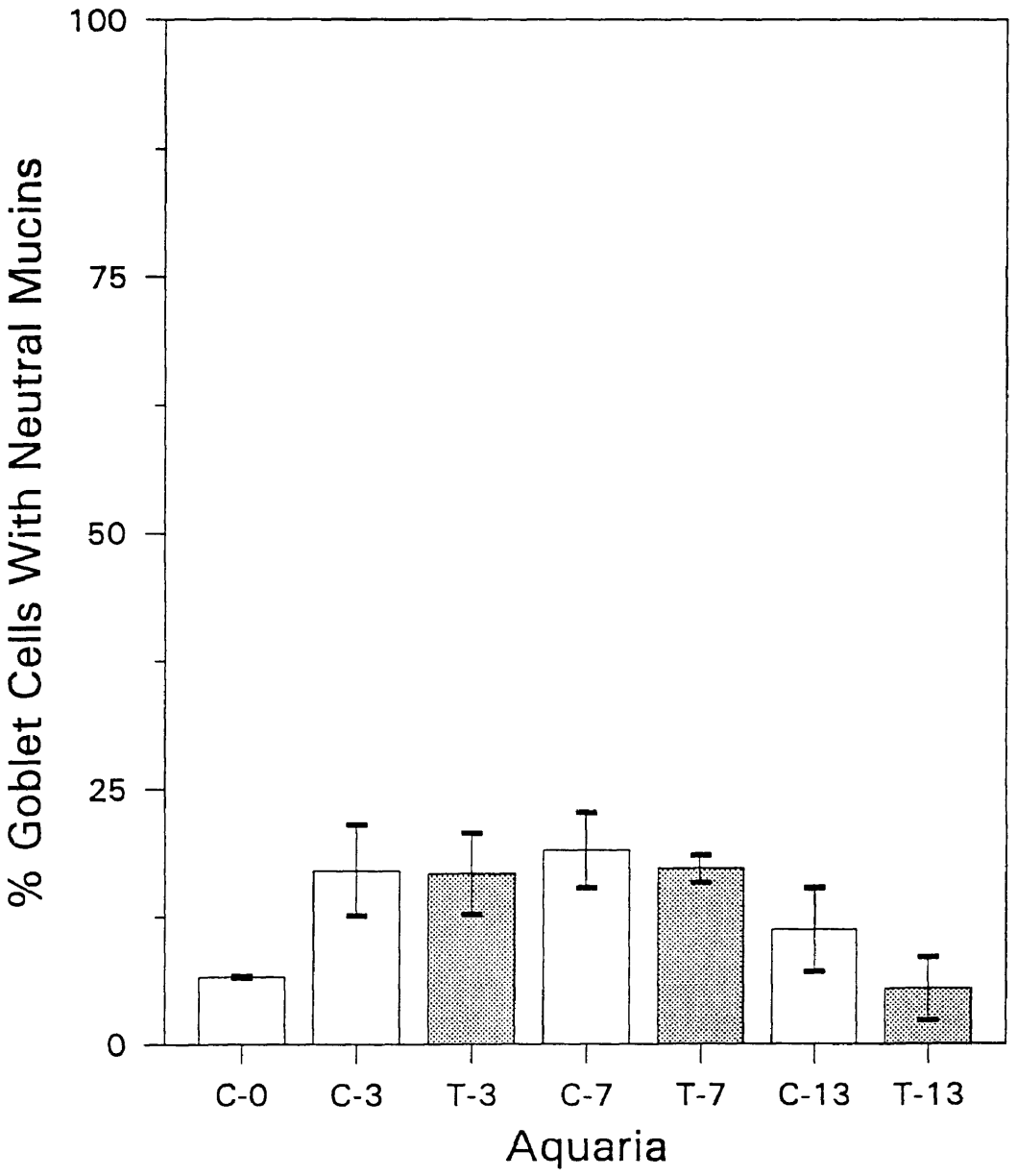
Neutral mucins:

The percentages of goblet cells containing neutral mucins increased from the C-0 levels (6.6% ± 0.1) to maximum values on Day 7 of 19.0% (± 3.7) for C-7 fish and 17.2% (± 1.4) for T-7 fish (Fig. 15). The percentages had decreased by Day 13, to C-0 values for T-13 fish (5.4% ± 3.1) though those of C-13 fish were still higher (11.2% ± 4.1). Control fish maintained slightly higher percentages than treated fish throughout the experimental period. The T-13 values were significantly lower ($p < 0.05$) than all others except C-0 and C-13 values.

Mixed-acid mucins:

The percentages of goblet cells containing mixed-acid mucins followed a trend opposite to that of the cells containing acid mucins (Fig. 16). Starting at 25.2% (± 5.2), their mean percentage increased to 54.0% (± 10.0) for C-3 fish and 46.4% (± 6.5) for T-3 fish. These values remained fairly constant throughout the rest of the experiment. The Tukey multiple comparison test did not indicate any significant difference at the 5% level between fish from the different aquaria.

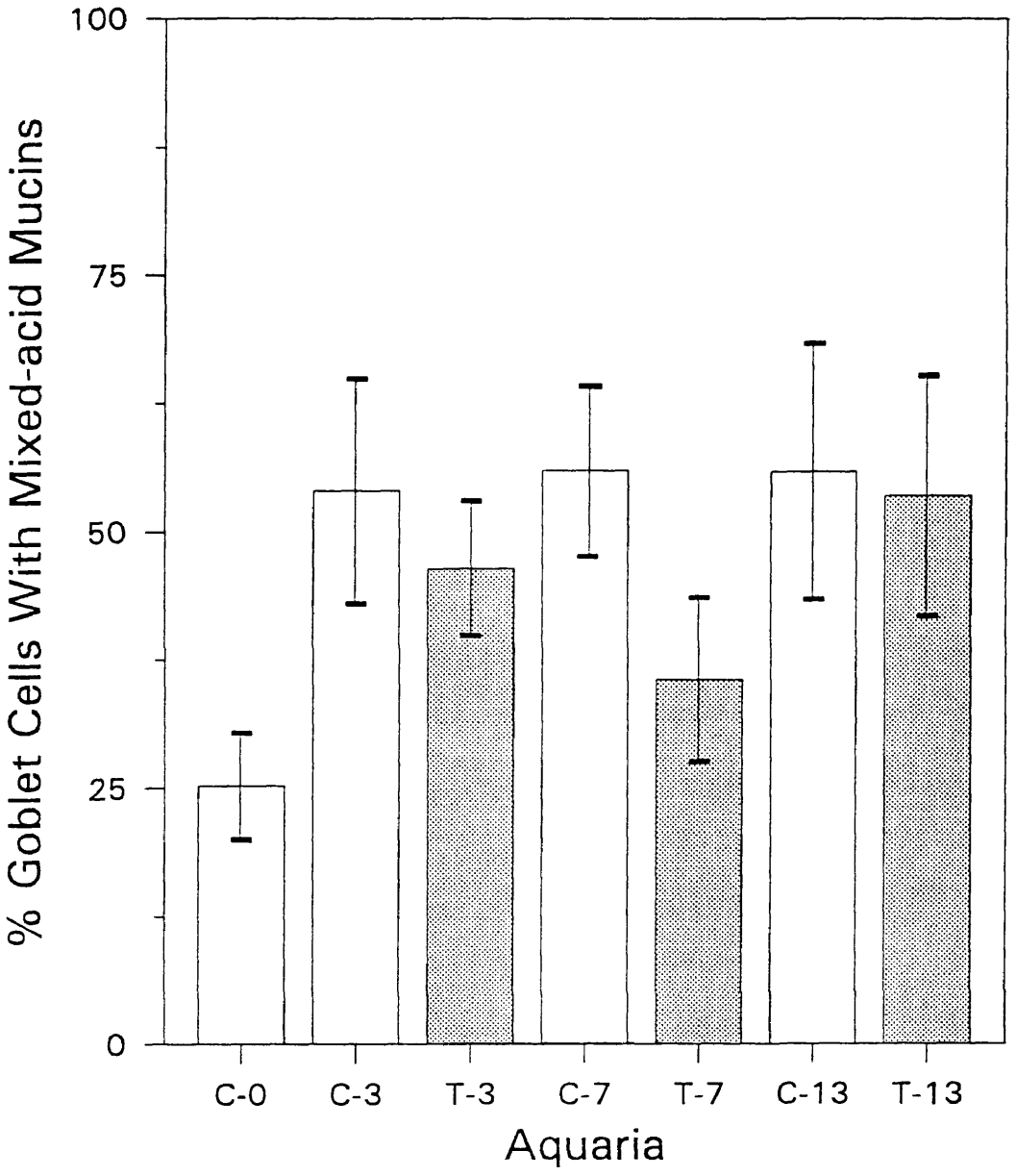
Figure 15. Percentage of epidermal goblet cells containing neutral mucins in mummichog, *Fundulus heteroclitus*, exposed to control and treatment (creosote-contaminated) sediment. Mean, standard deviation and Tukey multiple comparison test results (n=3, except for C-0 where n=2). Aquaria underlined by the same line are not significantly different ($p < 0.05$).



C-7 T-7 C-3 T-3 C-13 C-0 T-13

Tukey Multiple Comparison Test

Figure 16. Percentage of epidermal goblet cells containing mixed-acid mucins in mummichog, *Fundulus heteroclitus*, exposed to control and treatment (creosote-contaminated) sediment. Mean, standard deviation and Tukey multiple comparison test results (n=3, except for C-0 where n=2). Aquaria underlined by the same line are not significantly different ($p < 0.05$).



C-0 C-3 T-3 C-7 T-7 C-13 T-13

Tukey Multiple Comparison Test

Discussion

Exposure to contaminated Elizabeth River sediment has a wide range of effects on fish, as confirmed by this study. The contaminated sediment increased the Hb content of epidermal mucus, promoted development of epidermal lesions, decreased the condition index of *Fundulus heteroclitus* and induced mortality. The ER sediment appeared to stimulate mucus secretion and to decrease the thickness of the epidermis. Transfer of the fish to the experimental aquaria also proved to be an important, although temporary, factor in the observed changes.

In biological systems, environmental factors ancillary to the study may have a confounding effect and must be monitored. In this study, the water temperature, salinity and pH were found to be constant and within the normal range tolerated by mummichog (Abraham 1985). Therefore, these parameters are not considered to be a significant source of change in the epidermal parameters examined in mummichog, either between treatments or through time.

The DOC levels of the control aquaria were slightly, but significantly, higher than those of the treatment aquaria. This was unexpected since the treatment sediment actually has a slightly higher TOC level, but the result may be explained in terms of sediment resuspension. Butler *et al.* (1982) reported decreased activity in mummichog exposed to fuel oil. This was also true for the fish used in this study. Treated fish were less active than control fish, resulting in less resuspended sediment and a slightly

lower DOC concentration in the treatment aquaria.

The aromatic compound concentration in the water column underwent considerable change over the experimental period. On Day 2, the concentrations in the treatment aquarium effluents were substantially higher than on any other day. The decreases in concentrations seen on Days 6 and 12 were probably a result of the decline in resuspension of sediment by the fish. The aromatic compound concentration of the control aquarium effluent sampled on Day 6 was lower than those of treatment aquaria on any day. Fish exposure to aromatic compounds, however, depended on more than just their overall concentration in the water column. Measured concentrations of aromatic compounds in water subsequent to a vigorous extraction methodology are not an accurate measure of bioavailability (Varanasi 1989). The heavier, more hydrophobic compounds tend to associate with the sediment or DOM and therefore may be less biologically available than the lower molecular weight, more water soluble compounds (McCarthy and Jimenez 1985) which may have a proportionally greater contribution towards any deleterious effects. These lower molecular weight compounds were absent in the control aquarium effluent, reducing even more the potential exposure of control fish to aromatic compounds.

Weathering of the finite amount of aromatic compounds contained in the aquaria sediment, as well as their continuous flushing from the system could have caused a decrease in sediment toxicity. In fact, Roberts *et al.* (1989), exposing fish to ER sediment in another flow-through system, found evidence of reduced toxicity of the sediment after a 7-day flushing period. In the present study, there is no clear

evidence for a reduction of the aromatic compound concentration in sediment or for a change in relative constituent concentrations which would affect toxicity. Exposure of fish to aromatic compounds in the water column probably decreased with the decreased resuspension of sediment. This may have been off-set by greater contact with interstitial water, as the tendency of fish to remain on or very near the sediment increased. Burgess *et al.* (1993) showed that PCB toxicity was higher in suspended than in bedded exposures and that exposure to interstitial water was more toxic than either of them. The ventral skin of the treated fish, examined in this study, would have been subjected to the greatest exposure.

The significantly higher mortality in treatment aquaria was likely an effect of the ER sediment and may be attributable to the aromatic compounds therein. The actual cause of death cannot be determined for all fish. Some deaths were probably a direct result of the extensive ulcerations and resultant bleeding. Prasad and Kumari (1987) noted that coagulation of mucus on the gills of fish exposed to crude oil led to mortality. This may explain the death of fish in this study that did not have any apparent epidermal hemorrhage.

There is evidence for the imputation of aromatic compounds from ER sediment in lesion formation. Aromatic compounds such as PAHs and their metabolites have been positively correlated to the incidence of lesions in fish, including hepatic neoplasms (Malins *et al.* 1985; Krahn *et al.* 1986; Hawkins *et al.* 1991), possibly through the formation of DNA adducts (Van Der Oost *et al.* 1994). PAH metabolites

have been detected in various fishes from the Elizabeth River (Deshpande 1989) and hepatic neoplasms and other lesions are well known in mummichog from this river (Hargis *et al.* 1989; Vogelbein *et al.* 1990; Van Veld *et al.* 1992). Fin erosions, so abundant in this experiment, have been associated with mortality in fish exposed to crude oil (Khan 1987). The low density of goblet cells on fins (Pickering 1974) suggests that fins may lack a thick mucus protective layer. Frequent occurrence of this type of lesion is therefore not surprising.

The decrease in the condition index of treated fish provides a direct measure of the deterioration of their health due to the contaminated sediment. The decline in the CI at Day 3, observed in both the treated and the control fish, probably reflects the initial stress of netting the fish and their transport to smaller, turbid aquaria. Acclimation occurred for control fish by Day 7, and their CI had returned to initial values. The CI of treated fish did not change by Day 7, indicating a partial acclimation, but that stasis disappeared during the second week. The significant decrease in CI of the treated fish in the second week may be attributed to the presence of contaminants in the sediment. ER sediment may have interfered with the nutritional processes of the fish, perhaps by inducing a loss of appetite or by suppressing the nutrient uptake of ingested food. Hargis *et al.* (1984), Roberts *et al.* (1989) and Khan (1987) described a similar effect in fish exposed to ER sediment or crude oil.

Results of the Hb assay indicate that both the transfer of the fish to the

experimental aquaria and the contamination of the treatment sediment irritated the fish epidermis and probably contributed to the deterioration of fish health. An increase in hemoglobin in mucus is a non-specific response to tissue irritation or stress. Hemoglobin is released in the epidermis at the site of inflammation and transported with mucus (Speare and Mirsalimi 1992). The increased Hb concentration in epidermal mucus by Day 3 may be due to slight epidermal abrasions incurred during transfer to the experimental aquaria or to a general response to that transfer. The higher and ever increasing hemoglobin response in treated fish was most likely a function of the creosote content of the sediment.

Preliminary experiments failed to detect sialic acid in the mucus of *Fundulus heteroclitus.* Direct determination of mucus production and secretion was thus not possible. Therefore, mucus production was quantified by morphometric analysis of the epidermal goblet cells using light microscopy. The lack of statistically significant differences in the density or diameter of epidermal goblet cells from the separately stained (AB1.0/PAS and AB2.5/PAS) tissue samples of each fish indicate that the same goblet cell population was examined in the two different stains.

The appearance of the scales upon superficial examination of the skin samples from the fish exposed to treatment sediment for 13 days indicates a decrease in epidermal thickness. Similar reductions in epidermal thickness have been reported in fish exposed to copper (Iger *et al.* 1994), crude petroleum (Burton *et al.* 1984) and other environmental stressors (Iger *et al.* 1988). These reductions seem to be a result

of degenerative changes in the epidermis, such as necrosis of pavement and goblet cells. In the present experiment, the decrease in thickness could represent the initial lesion of the epidermis, leading to more severe ulcerations.

The decrease in goblet cell diameter over the experimental period points to increased mucus secretion. A decrease in goblet cell diameter may be indicative of recent mucus discharge (Battaglini *et al.* 1993). Prasad (1987) reported a temporary decrease in goblet cell diameter in fish exposed to crude oil extracts. In the present experiment, the decrease in goblet cell diameter on Day 3 is probably an effect of an increase in mucus secretion resulting from the transfer of the fish to the experimental aquaria. That decrease is temporary for control fish, demonstrating their acclimation to the aquaria. The continued decrease in goblet cell diameter of treated fish probably reflects the continued and excessive secretion of mucus elicited by exposure to the contaminated sediment. The decrease in thickness of the epidermis, seen in treated fish, could also partially account for the decrease in diameter, by decreasing the maturation time of the goblet cells. Evidence for epidermal thinning appears only in the second week, however, and is not sufficient to explain the decrease in goblet cell diameter throughout the experiment.

The drastic decline in the density of epidermal goblet cells in treated fish during the second week also suggests an increased mucus production. A temporary decrease in goblet cell density can be the result of an extensive release of mucus (Iger

and Abraham 1990). Such a trend, seen in C-3 fish, supports the possibility of a coordinated release of mucus by the goblet cells during or shortly after the transfer of the fish to the experimental aquaria.

The major change in goblet cell density, however, occurred in treated fish during the second week. The cell density of the T-13 fish decreased to a third of the C-0 value. This decline in goblet cell density and the continued decrease in diameter of the remaining goblet cells suggest an inability of treated fish to maintain the goblet cell production and mucus coverage necessary to protect them from contaminants in the treated sediment. Prasad (1987) observed a decrease in both goblet cell diameter and density in fish exposed to lethal extracts of crude oil, followed by a degeneration of the goblet cells in exposures longer than 12 hours. Longer exposures to contaminants may lead to lower goblet cell densities through different mechanisms. Increases in mucus production may facilitate ectoparasitic colonization (Goldes *et al.* 1988) which can cause a decrease in goblet cell density (Pottinger *et al.* 1984). Such infections might not have been detected in this study. The low condition index of T-13 fish, however, lends credence to a depletion of the energy reserves of treated fish and their inability to sustain the continued production of mucus.

Most studies (Mallatt 1985) report hyperplasia or hypertrophy of goblet cells as indicators of increased mucus production and as responses to numerous environmental stressors such as anionic detergents (Zaccone *et al.* 1985; Misra *et al.* 1987) and

metals (Mueller *et al.* 1991; Prasad and Shil 1993). Khan and Kiceniuk (1984) observed hyperplasia of goblet cells in fish exposed to crude oil. The lack of goblet cell hypertrophy and hyperplasia in this experiment was therefore an unexpected finding. Increased mucus production can occur without hypertrophy or hyperplasia, however, if the turnover rate of the goblet cells is increased. Decreased migration time of an increased number of goblet cells will decrease their mean diameter even as mucus secretion is increased. McDonald *et al.* (1991b) suggested an increase in mucus secretion to explain a decrease in sialic acid content - a major component of the mucus of certain fishes - in the gills of fish exposed to aluminum, a contaminant which usually results in hyperplasia and hypertrophy of goblet cells.

The percent mucin type produced by goblet cells did not seem to be affected by creosote-contamination of the sediment. There were no significant differences in the percent mucin type between goblet cells of control and treated fish on any individual day, with the exception of non-sulfated mucins on Day 13. This was a surprising result, considering the variety of environmental parameters that have been shown to induce a change in mucin types. Exposure to anionic detergents (Zaccone *et al.* 1985; Roy 1988), cadmium (Battaglini *et al.* 1993), ammonia (Ferguson *et al.* 1992) as well as changes in pH (Zuchelkowski *et al.* 1985) and salinity (Solanki and Benjamin 1982) have been observed to change the proportions of mucin types.

The change in mucin type seemed to be influenced by the transfer of the fish to the experimental aquaria. Though differences between treatments on a given day

were rare, the type of mucins produced changed significantly over time. The initial change in mucin types occurred at the start of the experiment, between Day 0 and Day 3, involving all types of mucins. The following ten days were marked mainly by a recovery in the proportions of goblet cells containing sulfated, non-sulfated, mixed-sulfated and neutral mucins to C-0 levels.

The turbidity of the water, which decreased over the experimental period, can probably be discounted as the stressor responsible for the change in mucin types. Ferguson *et al.* (1992) reported no change in mucin type in fish exposed to high concentrations of kaolin. A more likely stressor is the handling associated with the transfer of the fish to the aquaria. Increases in mucus production have been previously reported to occur as a result of handling (Pickering and Macey 1977). An extensive and sudden secretion of mucus at the start of the experiment, necessitating an increase in production, would likely result in the observed predominance of neutral, non-sulfated mucins, as they are easier to synthesize.

Conclusions

Exposure to creosote-contaminated Elizabeth River sediment had a profound and deleterious effect on the health of *Fundulus heteroclitus*.

Treated fish had a significantly lower condition index, higher occurrences of epidermal lesions, higher mortality and higher hemoglobin content in their mucus than control fish. There was also evidence for the thinning of the epidermis of treated fish, after two weeks of exposure. The epidermal goblet cell density of treated fish decreased by ca. 67% in the second week of exposure and the diameter of their goblet cells decreased significantly through time. These parameters were stable in control fish.

The decrease in diameter of the treated fish epidermal goblet cells suggest that mucus secretion was increased, by an increased turnover rate of goblet cells and mucus production. The significant decrease in goblet cell density observed on Day 13, the continued change in the mucus quality and the low condition index of the treated fish suggest a depletion of their energy reserves.

The initial transfer from the holding tank to the experimental aquaria was also a major source of stress for all fish. It resulted in a temporary decrease in condition index and increase in mucus hemoglobin content. The fish appeared to void their mucus cells in response to that stress, causing a decrease in both diameter and the density of epidermal goblet cells on Day 3. The higher rate of turnover of goblet

cells, necessary to replace that mucus, probably caused the reduction in the proportion of acid, sulfated mucins and an increase in the non-sulfated, neutral mucins. Control fish recovered from this initial stress and acclimated to experimental conditions. Treated fish showed a continued health deterioration.

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